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(54) Title: NUCLEIC ACID BIOSENSOR DIAGNOSTICS

(57) Abstract

A biosensor for direct analysis of nucleic acid hybridization by use of an optical fiber is disclosed. Single-stranded nucleic acid probes are immobilized onto the surface of optical fibers and undergo hybridization with complementary single-stranded nucleic acids introduced into the local environment of the sensor. Hybridization events are detected by the use of fluorescent compounds which intercalate into double-stranded nucleic acids. The invention finds uses in detection and screening of genetic disorders, viruses, and pathogenic microorganisms. Biotechnology applications include monitoring of cell cultures and gene expression. The invention includes biosensors systems in which fluorescent molecules are tethered to immobilized single-stranded nucleic acid. The preferred method for immobilization of single-stranded nucleic acids is by *in situ* solid phase nucleic acid synthesis.

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NUCLEIC ACID BIOSENSOR DIAGNOSTICS

Background of the Invention

The present invention is directed generally to biosensors that are useful in the identification and analysis of biologically significant nucleic acids. The biosensors of the present invention and their applied methods provide a means for the direct analysis of nucleic acid hybridization and, therefore, have application to a myriad of biological fields including clinical diagnostics.

The detection and identification of microorganisms is a problem common to many areas of human and veterinary health. For example, the detection of pathogenic species such as Salmonella typhimurium, Listeria monocytogenes, and Escherichia coli, which are causative agents of major food borne epidemics, is a great concern within the food industry with respect to the quality and safety of the food supply.

In other areas of human and veterinary health care, detection and identification of infectious diseases caused by pathogenic microorganisms and viruses is a first step in diagnosis and treatment. For example, it is estimated that 10-15 million office visits per year are for the detection and treatment of three major pathogens - Chlamydia ssp., Trichomonas vaginalis and Gradenerella vaginitis. Infections of these organisms annually effect 3.75 million, 0.75 million and 1.5 million patients, respectively.

Classical techniques routinely used for the detection and identification of microorganisms are often labor intensive involving plating procedures which require lengthy analysis times. To illustrate, the method currently employed for the detection of Listeria monocytogenes in food and feed commodities

involves a three stage analysis. The analysis begins with enrichment of the sample to be analyzed in a nutrient broth for 2 to 4 days. After the enrichment period, plating of the sample onto selective agar media is done and the sample is allowed to incubate for 2 days in order to obtain colonies for biotyping and serotyping, which may take as long as 20 days to complete (McLauchlin et al., 1988, Microbiology Review, 55: 578).

Detection processes based on culturing require analysis times which are too lengthy for effective monitoring and timely intervention to prevent the spread of biohazardous materials or treat disease. In addition, although these methods have been improved over the last decade, the chance of obtaining false negative results is still considerable, and many microorganisms are difficult to culture. Thus, plating/culture methods are limited with respect to their sensitivity, specificity, and lengthy analysis times that are required.

In order to shorten the time required to detect and identify pathogenic bacteria, viruses and genetic diseases, rapid tests such as enzyme immunoassays (EIA) have been developed (Olapedo et al., 1992). Although immunoassay techniques can be very sensitive and effective, there are practical drawbacks which have restricted the use of these methods. Such drawbacks include the need for highly skilled personnel, lengthy analysis and preparation times, and the large quantities of costly reagents that are required to do such analysis.

With the advent of nucleic acid amplification techniques (the polymerase chain reaction), the in vitro amplification of specific sequences from a portion of DNA or RNA is now possible. Detection of very low numbers of microorganisms has been demonstrated (Rossen et al., 1991; Golsteyn et al., 1991; Wernars, K., et al., 1991). The polymerase chain reaction technique is sensitive and specific but involves complex manipulations in carrying out the tests and is not particularly well-suited for large numbers of samples.

Due to the sensitivity of PCR technology, special rooms or areas for sample preparation and analysis are required to prevent contamination. In many tests PCR results must be confirmed by additional hybridization analysis. RNAs are difficult to assay by PCR but are very important for human viral detection. In general PCR needs to be automated for acceptance as a practical diagnostic tool. Hybridization methods require as much as three or four days to complete results. Although the actual hybridization step can be as short as 18 hours, the entire detection process of a DNA/DNA hybrid can take as long as three days with a radioisotope marker.

Thus, there is a great need for simpler, faster and more cost-effective means for detecting specific biologically important RNA and DNA sequences in the fields of human and veterinary in vitro diagnostics, food microbiology, and forensic applications.

Biosensors developed to date begin to overcome drawbacks associated with the current state of the art in detecting and identifying microorganisms. A biosensor is a device which consists of a biologically active material connected to a transducer that converts a selective biochemical reaction into a measurable analytical signal (Thompson et al., 1984, Trends in Analytical Chemistry, 3: 173; Guilbault, 1991, Current Opinion in Biotechnology, 2: 3). The advantages offered by biosensors over other forms of analysis include the ease of use (by non-expert personnel), low cost, ease of fabrication, small size, ruggedness, facile interfacing with computers, low detection limits, high sensitivity, high selectivity, rapid response, and reusability of the devices.

Biosensors have been used to selectively detect cells, viruses, other biologically significant materials, biochemical reactions and immunological reactions by using detection strategies that involve immobilization of enzymes, antibodies or other selective proteins onto solid substrates such as quartz

(for piezoelectric and optical sensors) or metal (for electrochemical sensors) (Anrade et al., 1990, Biosensor Technology: Fundamentals and Applications, R.P. Buck, W.E. Hatfield, M. Umaña, E.F. Bowden, Eds., Marcel Dekker Inc., NY, pp. 219; Wise, 1990, Bioinstrumentation: Research, Developments and Applications, Butterworth Publishers, Stoneham, MA). However, such sensors are not widely available from commercial sources due to problems associated with the long-term stability of the selective recognition elements when immobilized onto solid surfaces (Kallury et al., 1992, Analytical Chemistry, 64: 1062; Krull et al., 1991, Journal of Electron Microscopy Technique, 18: 212).

An alternative approach is to create biosensors with long-term chemical stability. One such approach takes advantage of the stability of DNA. With the recent advent of DNA probe technology, a number of selective oligomers which interact with the DNA of important biological species, for instance salmonella, have been identified (Symons, 1989, Nucleic Acid Probes, CRC Press, Boca Raton, FL; Bock et al., 1992, Nature, 355: 564; Tay et al., 1992, Oral Microbiology and Immunology, 7: 344; Sherman et al., 1993, Bioorganic & Medicinal Chemistry Letters, 3: 469). These have been used to provide a new type of biorecognition element which is highly selective, stable, and can be easily synthesized in the laboratory (Letsinger et al., 1976, Journal of the American Chemical Society, 98: 3655; Beaucage et al., 1981, Tetrahedron Letters, 22: 1859; Alvarado-Urbina et al., 1981, Science, 214: 270) as compared to other chemically synthesized biorecognition elements, such as catalytic antibodies (Shokat et al., 1993, Methods in Enzymology, 203: 327). As a result, species-specific DNA probes may now be exploited for biosensor development. DNA biosensors that are presently being developed are largely based on piezoelectric and electrochemical transducers and more recently optical transducers.

Two strategies have been employed for piezoelectric biosensors that use DNA. Both strategies begin by immobilizing

probe DNA onto the surface of a piezoelectric crystal. The immobilized probe is then allowed to hybridize to target DNA that is introduced to the local environment of the sensor. The first approach relies on observing a change in the resonance frequency of the crystal as a function of a significant mass increase at the crystal surface as given by the Sauerbrey equation (Downs et al, 1987, Analytical Letters, 20: 1897). This method works provided that the target strands are sufficiently large (>900 nucleotides) (Down et al., 1987, Analytical Letters, 20: 1897; Andle et al., 1992, Sensors and Actuators B., 8: 191), or that a high mass metallic chelator may be attached to the target strands to ensure a detectable mass change at the surface of the crystal (Richards et al., European Patent Application, EP295965 A2, December 21, 1988).

A second approach involves the simultaneous monitoring of changes in interfacial properties at the surface of the crystal such as microviscosity, elastic modulus, and dielectric constant, based on network analysis permitting detection of shorter oligomers than piezoelectric sensors (Su et al., 1993, Analyst, 118: 309). Sensors which respond to interfacial mass changes have been able to detect 1 ng of complement DNA (cDNA), while 0.3 mg of CDNA could be detected by network analysis techniques. The major limitations faced by piezoelectric sensors include non-selective adsorption, surface occlusion, and the inability of the sensor to identify the selective binding process over the multitude of dynamic binding events that occur at the interface (Thompson et al., 1991, Analytical Chemistry, 63: 393A).

Voltammetric detection of DNA on the surface of oxidized glassy carbon electrodes has been investigated by Mikkelsen and co-workers (Millan et al., 76th Canadian Society for Chemistry Conference and Exhibition; Millan et al., 1993, Analytical Chemistry, 65: 2317). This was accomplished by selectively immobilizing thymidylic acid icosanucleotide (dT_{20}) capture probes terminated with a poly-dG tail onto the surface

of a glassy carbon electrode activated with 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide via the dG residues. Detection of target-probe hybridization was achieved by cyclic voltammetry in conjunction with the redox active metallo-intercalators tris (2,2'-bipyridyl) cobalt(III) perchlorate ($Co(bpy)_3^{3+}$) and tris (1,10-phenanthroline) cobalt(III) perchlorate ($Co(phen)_3^{3+}$). Although the limit of detection and sensitivity of the device were not stated by the authors, a concentration of 0.12 mM of the electro-active hybridization indicator was required to provide an observable increase in the cathodic peaks of cyclic voltammograms obtained before and after hybridization had occurred. The problems faced by this system, as with all electrochemical detection schemes, include: electrical noise, nonselective adsorption and surface occlusion leading to drift of binding activity, evolution of mixed potentials, and spurious voltammetric peaks (Thompson et al., 1991, Analytical Chemistry, 63: 393A).

The use of a redox-active intercalator, which is known to associate only with double stranded DNA, ensures that the observed signal is indeed from the selective binding interaction. In this way the problem of identifying the chemical origin of binding events found with piezoelectric sensors was overcome. However, a limitation specific to this electrochemical method lies in the procedure used to immobilize the DNA probe. The carbodiimide condensation which links the amine moieties of the poly-dG tail of the probe to the surface of the glassy carbon electrode restricts the composition of the probe to one which contains only thymine. Inclusion of the other nucleotide bases (A,C,G) in the probe sequence would result in immobilization of probes onto the electrode surface via reactive groups in non-thymine nucleotides, thereby restricting the ability of the probe to hybridize with target strands and reducing the selectivity of the probe.

Summary of the Invention

The present invention concerns biosensors for direct detection of nucleic acids and nucleic acid analogs, and relies upon fluorescence that occurs when a fluorophore intercalates with a hybridized nucleic acid duplex. The subject invention uses an optical wave guide, such as an optical fiber, onto which single-stranded nucleic acids or nucleic acid analogs have been immobilized. Target single-stranded nucleic acids hybridize with the immobilized single-stranded nucleic acids; a fluorophore then intercalates in such hybridized duplex. Upon excitation by a light source, a fluorescence signal is produced and detected to indicate the presence of the target single-stranded nucleic acid.

In first embodiments of this invention, the fluorophore is in a solution environment surrounding the immobilized single-stranded nucleic acid.

In second embodiments, the fluorophore is tethered to the immobilized single-stranded DNA, for example by use of a hydrocarbon tether. The use of tethered probes can significantly reduce biosensor response time. A further embodiment allows for the simultaneous detection of a plurality of types of target single-stranded nucleic acids by providing a plurality of immobilized single-stranded nucleic acid with a plurality of tethered fluorophores.

The present invention provides biosensors for direct analysis of nucleic acid hybridization by use of an optical substrate such as an optical wafer or an optical fiber, and single-stranded nucleic acids or nucleic acid analogs which have been immobilized onto the optical substrate. Generation of a fluorescence signal upon hybridization to complementary nucleic acids and nucleic acid analogs in a sample may be achieved in a number of different ways. Biosensors of this invention are sufficiently sensitive to directly detect sub-nanomole quantities of target nucleic acids in a sample without the need to employ nucleic acid amplification techniques such as PCR techniques.

Biosensors of this invention can have detection limits for target nucleic acids down to several femtomoles or less.

Optical biosensor comprise single-stranded nucleic acid or nucleic acid analogs of a specific selected sequence immobilized onto activated optical supports. The selected immobilized sequences are capable of binding to target sequences, including sequences characteristic of or selective for viruses, bacteria, or other microorganisms as well as of genetic disorders or other conditions. Biosensors having such characteristic or selective immobilized sequences are useful for the rapid screening of genetic disorders, viruses, pathogenic bacteria and in biotechnology applications such as the monitoring of cell cultures and gene expression. Activated optical supports include optical fibers or wave guides among others.

The biosensor system of the present invention detects a target nucleic acid, and comprises a light source, a detector, and an optical element for receiving light from the source and conveying it to an interaction surface of the optical element. A single-stranded nucleic acid or nucleic acid analog, for a particular nucleic acid sequence which is complementary to the target nucleic acid, is immobilized onto the interaction surface of the optical element. Fluorescent molecules are provided that will intercalate upon hybridization of the single-strand nucleic acid with the complementary target nucleic acid and fluoresce when stimulated by the light source. The fluorescence is conducted by the optical element to the detector, indicates that the target nucleic acid has hybridized to the immobilized probe and thus detects the presence of the target in the sample. An interaction surface is defined to mean a surface of the optical element on which single-stranded nucleic acid is immobilized, and at which the fluorescent molecules interact with the light.

In specific embodiments, preferred high-sensitivity (low-detection limit) biosensors are provided by activating the interaction surface of an optical element with linkers of at

least about 25Å (Angstrom) in length and attachment of a selected probe nucleic acid sequence to that linker. (A probe nucleic acid is, at least in part, complementary to a target nucleic acid.) The preferred method for attachment of the probe nucleic acid to the linker is by in situ synthesis of the nucleic acid sequence onto the linker using solid-phase nucleic acid synthesis methods or routine modifications of those methods. Such methods of in situ synthesis are particularly useful for immobilization of nucleic acids of 50 or fewer bases and more particularly useful for nucleic acids of 30 or fewer bases.

In another specific embodiment, this invention provides biosensors in which the interaction surface is treated to immobilize selected nucleic acid probe sequence such that the immobilized layer (including any linking molecules) has an index of refraction substantially equal to (i.e. within about ±10% of) the index of refraction of the surface of the optical element to which the layer is immobilized. In such biosensors, fluorophores in the immobilized layer will generally experience higher intensities of exacting light leading to higher enacted fluorescence intensities. Such biosensors will generally have increased sensitivity for use of longer nucleic acid probes. In particular, such biosensors will have increased sensitivity for use of nucleic acid probes of 120 or more bases. The index of refraction of the immobilized layer is dependent, at least in part, on the loading of immobilized molecules and linkers on the surface and the chemical nature of the immobilized molecules and any linkers.

In greater detail, the optical element preferably comprises an optical waveguide which also conveys the fluorescent light to the detector. The optical waveguide preferably conveys the emitted light by total internal reflection to the interaction surface of the optical waveguide. The optical waveguide can comprise an optical fiber, a channel waveguide, or a substrate that confines light by total internal reflection. The fluorescent molecules preferably exhibit sufficient Stokes shift

such that the wavelength of the light source and the wavelength of the fluorescent light are separated. The fluorescent molecules can be provided in a solution in which the optical element is immersed, or can be bound by a tether to the single-stranded nucleic acid.

The biosensor system of the present invention can be constructed and used to detect each of a mixture of target single-stranded nucleic acids (for example, Chlamydia and Gonorrhea in urogenital infections or E. coli and Salmonella during food processing) by using a plurality of fluorophores, each of which is tethered to an immobilized single-strand nucleic acid that is characteristic of or specific for detection of a given species or strain.

Accordingly, such a biosensor can also include a plurality of single-stranded nucleic acids, each having a tethered fluorophore which fluoresceses at a different wavelength, each having a distinct nucleic acid sequence, and each of which is complementary to a selected target nucleic acid. Fluorescence wavelength will then be specific for hybridization of a given target nucleic acid to its complementary immobilized probe.

The present invention also provides a recyclable or disposable biosensor for detecting a target nucleic acid, which biosensor includes an optical element for receiving and conveying light to an interaction surface of the optical element and single-stranded nucleic acid, for a particular nucleic acid sequence which is complementary to the target nucleic acid, immobilized onto the interaction surface of the optical element. The recyclable or disposable biosensor preferably comprises an optical waveguide, which preferably conveys the light by total internal reflection to the interaction surface of the optical waveguide. The optical waveguide preferably comprises an optical fiber. Fluorescent molecules are provided in a solution in which the recyclable or disposable biosensor is immersed that will

intercalate upon hybridization of the single-stranded nucleic acid with complementary target nucleic acid and fluoresce when stimulated by light. Alternatively, the fluorescent molecules are provided bound by a tether to the single-stranded nucleic acid.

The present invention also provides a method for detecting a target nucleic acid by providing an optical element with single-stranded nucleic acid, having a particular nucleic acid sequence which is complementary to the target nucleic acid, immobilized onto an interaction surface of the optical element, providing fluorescent molecules in proximity to the interaction surface of the optical element which will intercalate upon hybridization of the single-stranded nucleic acid with the complementary target nucleic acid and fluoresce when stimulated by light, illuminating the fluorescent molecules with light from a light source, and detecting fluorescent light from the fluorescent molecules, which indicates detection of the target nucleic acid.

In the practice of the present invention, the light source can be any suitable source such as a gas laser, solid state laser, semiconductor laser, a light emitting diode, or white light source. The detector can be any suitable detector such as a photomultiplier tube, an avalanche photodiode, an image intensifier, multichannel plate, or semiconductor detector. The biosensor system can be a multiwavelength, multifluorescent system. The light coupling of the system can also be modified to allow a multitude of disposable biosensors to be analyzed either sequentially or in parallel.

The biosensors of the present invention have an improved detection limit and sensitivity and are shown to be stable over prolonged storage and severe washing conditions. Biosensors of this invention also allow for more rapid sample analysis with improved response time for signal generation.

The invention accordingly will allow for the rapid screening of genetic disorders, viruses and pathogenic microorganisms in prepared samples. The invention is also useful for monitoring of cell cultures for various biotechnology applications. The present invention also contemplates applications in human and veterinary in vitro diagnostics, food microbiology and forensics.

Brief Description of the Drawings

Figure 1 is a schematic representation of a biosensor system in which light from a suitable source is directed through a dichroic mirror beam splitter onto a fiber or waveguide coupler and then into an optical fiber having single-stranded nucleic acid bound to the surface thereof, and in which any resultant fluorescent light travels back through the coupler, and reflects off the beam splitter onto a detector, the output of which is digitized and directed to a computer for analysis thereof;

Figure 2 is a diagram of one embodiment of an apparatus used to measure fluorescence intensity from optical fibers coated with immobilized DNA;

Figure 3 illustrates a synthetic scheme used to activate the surface of the optical fibers with long chain aliphatic spacer molecules terminated with a 5′-O-dimethoxytrityl-2′-deoxythimine nucleoside;

Figure 4 is a graph showing response characteristics of a DNA optical biosensor immediately after preparation;

Figure 5 is a graph showing response characteristics of a DNA optical biosensor after storage for one month;

Figure 6 is a graph showing relative fluorescence intensity plotted against c-DNA concentration; and

Figure 7a illustrates a further embodiment in which a tether is attached to the free end of the single-stranded nucleic acid, so that a fluorophore can be attached to each single-stranded nucleic acid strand, and Figure 7b illustrates the fluorophore in solution.

Figure 8 illustrates a synthetic scheme for preparation of a tethered ethidium analogue for attachment to immobilized oligonucleotides.

Figure 9 is a graph showing response time of a reagentless biosensor as described in Example 10. The graph measures fluorescence of a tethered dye as a function of time after exposure to a sample of 720 ng of cDNA.

Detailed Description of the Invention

The present invention describes biosensors for direct detection of nucleic acids and nucleic acid analogs. It relies on fluorescence that occurs when a fluorophore intercalates with a hybridized nucleic acid duplex. The invention uses an optical wave guide, such as optical fiber, onto which single-stranded nucleic acids or nucleic acid analogs have been immobilized.

Single-stranded nucleic acid is covalently immobilized onto optical fibers by first activating the surface of the optical fiber with a long chain aliphatic spacer arm terminated by a 5′-O-dimethyoxytrityl-2′-deoxyribonucleotide, followed by automated solid-phase DNA synthesis. Detection of double stranded nucleic acids or nucleic acid analogs at the fiber surface after hybridization between immobilized single-stranded nucleic acid and its complementary single-stranded nucleic acid is achieved by measuring enhanced fluorescence emission of the intercalated fluorophore.

The configuration of the described biosensor can include immobilized nucleic acids (DNA and RNA), modified nucleic

acids, and nucleic acid analogs prepared by well-known methods or by straight-forward extension or modification of those methods. The term nucleic acid includes polynucleotides, oligomers, relatively short polynucleotides (up to about 50 bases) and longer polynucleotides ranging up to several hundred bases. There is no specific size limit on nucleic acids used for immobilization in this invention. However, problems due to self-hybridization may occur with longer nucleic acids. As used herein, the term "nucleic acid analogs" includes modified nucleic acids. As used herein, the term "nucleotide acid analog" includes nucleic acids where the internucleotide phosphodiester bond of DNA or RNA is modified to enhance bio-stability of the oligomer and "tune" the selectivity/specificity for target molecules (Ulhmann, et al., 1990, Angew. Chem. Int. Ed. Eng., 90: 543; Goodchild, 1990, J. Bioconjugate Chem., 1: 165; Englisch et al., 1991, Angew, Chem. Int. Ed. Eng., 30: 613). Such modifications may include phosphorothioates, phosphorodithioates, phosphotriesters, phosphoramidates or methylphosphonates.

RNA may be assembled on the support or prepared separately and linked to the support post-synthesis. RNA monomers are commercially available, as are some 2′-O-modified synthons. The 2′-O-methyl, allyl and 2′-deoxy-2′-fluoro RNA analogs, when incorporated into an oligomer show increased biostability and stabilization of the RNA/DNA duplex (Lesnik, et al., 1993, Biochemistry, 32: 7832).

As used herein, the term "nucleic acid analogs" also include alpha anomers (α-DNA), L-DNA (mirror image DNA), 2′-5′ linked RNA, branched DNA/RNA or chimeras of natural DNA or RNA and the above-modified nucleic acids. Back-bone replaced nucleic acid analogs can also be adapted to use in the biosensor of the present invention.

For purposes of the present invention, the peptide nucleic acids (PNAs) (Nielsen et al., 1993, Anti-Cancer Drug Design, 8: 53; Engels et al, 1992, Angew, Chem. Int. Ed. Eng.,

31: 1008) and carbamate-bridged morpholino-type oligonucleotide analogs (Burger, D.R., 1993, J. Clinical Immunoassay, 16: 224; Uhlmann, et al., 1993, Methods in Molecular Biology, 20, "Protocols for Oligonucleotides and Analogs," ed. Sudhir Agarwal, Humana Press, NJ, U.S.A., pp. 335-389) are also embraced by the term "nucleic acid analog." Both exhibit sequence specific binding to DNA with the resulting duplexes being more thermally stable than the natural DNA/DNA duplex. Other back-bone replaced nucleic acids are well-known to those skilled in the art and may also be used in the present invention (See e.g., Uhlmann et al., 1993, Methods in Molecular Biology, 20, "Protocols for Oligonucleotides and Analogs," ed. Sudhir Agrawal, Humana Press, NJ, U.S.A., pp. 335).

The biosensor of the present invention provides rapid clinical testing for viruses (e.g., HIV, T cell lymphotropic virus 1 and 2, hepatitis B and C), and pathogenic bacteria (e.g., E. coli., Salmonella, Listeria, Chlamydia ssp., Trichomonas vaginalis, Gradenerella vaginitis) as well as other microorganisms. Detection of genetic disorders (e.g., cystic fibrosis and sickle-cell anemia) and diseases such as cancer is also contemplated by the method and apparatus of the present invention.

In the present invention, changes in fluorescence emission are based on intercalation of environmentally sensitive fluorophores, such as ethidium bromide or dipyridophenazine complexes of ruthenium (Hafeman et al., 1988, Science, 240: 1182). The fluorophore is available in the local environment of the immobilized single-stranded nucleic acid, for example by being in solution or by being tethered to the immobilized single-stranded nucleic acid. Upon hybridization of the immobilized single-stranded nucleic acid with the target single-stranded nucleic acid, the intercalated fluorophore is activated. The intercalated fluorophore absorbs the light directed by the waveguide and re-emits the light in the form of fluorescence.

The fluorescent intensity is directly proportional to the concentration of target single-strand nucleic acid present.

The present invention utilizes the fluorescence response of the intercalated fluorophore and monitors a total internal reflection configuration along an optical fiber to quantify the presence of double stranded nucleic acids at the surface of the fiber. The fluorescence intensity is directly proportional to the amount of target nucleic acid or nucleic acid analog initially present in solution.

Fluorescence is the analytical method chosen for the transduction of hybridization events into a measurable analytical signal, since fluorescence techniques have long been known to provide high sensitivity (comparable to radioisotopic methods) and detailed information about structure at the molecular level (Lakowicz, 1983, Principles of Fluorescence Spectroscopy, Plenum Press, NY). Changes in the polarity, pH, temperature, microviscosity, or orientation of molecules in the local environment of a fluorophore may result in alteration of the electronic structure or collisional probabilities of the fluorophore. Such environmental changes may be detected by monitoring fluorescent signal parameters such as intensity, wavelength, lifetime, or polarization.

The fluorophore of the present invention can be for example ethidium bromide (EB). The ethidium cation (3,8-diamino-6-phenyl-5-ethyl-phenanthridium) is a fluorescent compound which strongly associates with double stranded nucleic acids by intercalation into the base-stacking region and, in some cases, the major groove of the double helical structure (Monaco et al., 1993, Journal of Bimolecular Structure and Dynamics, 10: 675). The ethidium cation is particularly well suited for investigations of nucleic acid hybridization for a number of reasons. Firstly, the quantum yield of the dye is known to increase as much as 100-fold when intercalated into the base stacking region (Bauer et al., 1989, Proceedings of the National

Academy of Science USA, 56: 7937). Secondly, the binding affinity and the fluorescence enhancement of the dye are independent of base composition (Cuniberti et al., 1990, Biophysical Chemistry, 38: 11). Thirdly, intercalation of the ethidium cation is known to increase duplex stability as the two 3,8-amino substituents hydrogen bond with the internucleotide phosphate groups on each of the DNA strands (whereas other intercalators are known to significantly decrease duplex stability) (Cuniberti et al., 1990, Biophysical Chemistry, 38: 11). Fourthly, EB has an absorption maximum of 510 nm, which is sufficiently close to the output wavelength of 488 nm of the Ar laser used in a fluorescence microscope to excite the fluorophore. The dye has an emission maximum of 595 nm when bound to DNA which is a sufficiently large Stoke's shift to make separation of the emission radiation from the excitation radiation straight forward, and to prevent inner filter effects, by the use of a dichroic mirror (Haugland, 1992, Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals, 5th Ed., USA: Molecular Probes Inc.). Due to the above mentioned reasons, the use of EB has been shown to provide a sensitive means to detect the presence of nucleic acid duplexes for this application.

Other examples of classes of fluorophores which can be used in the present invention include acridine dyes, phenanthrides, phenazines, phenothiazines, quinolines, aflatoxin, polycyclic hydrocarbons, oxirane derivatives, actinomyces, anthracyclinones, thiaxanthenones, anthramycin, mitomycin, platinum complexes, polyintervalators, norphilin A, fluorenes and fluorenones, furocoumarins, benzodipyrones and monostral fast blue.

In one embodiment of the invention, a tethered amine, alcohol, acid, thiol or other functionality is attached to the free, 5'-end or 3'-end of the immobilized single-stranded nucleic acid so that a fluorophore can be attached to each single strand. A schematic of this is shown in Figure 7a. In this embodiment,

a tether terminated with a primary amine moiety is attached to the 5'-end of the oligonucleotide and used to label the terminal end of the immobilized oligomer with a fluorescent probe in such a manner so as not to interfere with the ability of the nucleic acid strands to hybridize. For the case where the nucleotides are grown on the support via solid phase phosphoramidite synthesis, this can readily be achieved through the use of the commercially available reagent Aminolink 2®.

The fluorophore attached to the oligonucleotide via the tether may be the "molecular light switch" $Ru(phen)_2dppzPF_6$ developed by Jenkins et al. (1992, J. Amer. Chem. Soc. 114: 8736). This fluorescent probe is chosen as it is an example of an excellent fluorescent probe which is quenched (non-emissive) when in the presence of single-stranded nucleic acids and provides intense luminescence when in the presence of double stranded nucleic acids. This change in observed luminescence occurs via changes in the relative rates of radiative and non-radiative relaxation processes of the probe when the external environment changes from aqueous solution to a hydrophobic and highly structured one in the base stacking region of double stranded nucleic acids.

A specific example of a tethered fluorophore is illustrated in the synthetic scheme of Figure 8. In this case a modified ethidium-type dye with tether, here a C_{13} acid moiety, is synthesized as shown. The ethidium analogue with acid tether is attached to 5'-hexylamine functionalized oligonucleotides immobilized on the surface of an optical fiber to generate the biosensor with the tethered fluorophore probe.

Optical substrates such as planar wafers and optical fibers may be used as membrane-based sensors in the present invention. A preferred embodiment utilizes optical fibers. Optical fibers are particularly advantageous as membrane supports due to their small size, high light transmission capability, and ability to allow total internal reflection (TIR) of light which

can produce evanescent electric fields. Optical fibers also provide a compact and rugged sensing device, and offer the ability to do remote spectroscopic measurements (Love et al., 1991, Biosensors with Fiberoptics, D.L. Wise and L.B. Wingard (Eds.), Humana, NJ, pp. 139-180).

There are two fundamental configurations in which alterations in fluorescence parameters from fluorescently doped membranes on optical fibers may be monitored, namely, extrinsic mode and intrinsic mode. Extrinsic mode configurations are those in which the waveguide is simply used as a light pipe or conduit. End-on extrinsic mode investigations are usually done using optical fibers. In a biosensor which uses end-on extrinsic mode configurations, the fluorescent dyes and selective chemistry are located on or near the distal end of the fiber. The fiber is used as a light-pipe or conduit, where the excitation or emission radiation is simply guided from the sampling region to the detector. Fluorescence is stimulated by coupling excitation radiation into the near end of a fiber, and emission can be monitored by placing light sensing equipment directly opposite the distal end of the fiber.

Alternatively, the detector is placed at the near end of the fiber as some of the fluorescence may be coupled back into the fiber and totally internally reflected back to the near end. The side-on extrinsic mode approach is typically used for investigations carried out on planar supports, but may also be used for fibers. The immobilized single-strand nucleic acid and fluorophore are placed along the length of the optical fiber waveguide/wafer. The fiber is illuminated by a light source located normal to the length of the fiber and fluorescence emission is also monitored by equipment placed normal to the fiber. Extrinsic configurations provide the advantage that simple and inexpensive equipment, including conventional light sources and detectors, are used (Krull et al., 1991, Fiber Optic Chemical Sensors and Biosensors, Vol. II, O.S. Wolfbeis, Ed., CRC Press, Boca Raton, pp. 315). However, the extrinsic sampling

configuration provides poorer sensitivity owing to the short path length and sensitivity to interferents present in the surrounding media. In a preferred embodiment, an intrinsic mode arrangement is used to monitor fluorescence emission from the surface of optical fibers.

Fluorophores present at either the surface or just below the surface of the fiber may be excited through the formation of a standing wave electric field which propagates normal to the surface of the fiber upon total internal reflection of radiation in the fiber. The process of TIR occurs when the angle of reflection, θ, at the interface between a fiber of high refractive index, n_1, and the external medium of lower refractive index, n_2, is larger than a critical angle, θ_c, defined as:

$$\sin \theta_c = \frac{n_2}{n_1}$$

The amplitude of the electric field of the reflecting radiation decreases exponentially as a standing wave into the medium having the lower refractive index. This decaying radiation is referred to as an evanescent wave and can be used to excite fluorophores located near the boundary for TIR. The propagation intensity, I, of the evanescent wave depends on the reflection angle, θ, the wavelength of the transmitted radiation, λ, and a Fresnel transmission factor, T:

$$I = T(\theta)\exp(-2x/d_p)$$

where x represents distance normal to the boundary for TIR, and d_p is the penetration depth which is given by (Krull <u>et al</u>., 1990, Talanta, <u>37</u>: 801-807):

$$d_p = \frac{\lambda}{4\pi\,(n_1^2\sin^2(\theta) - n_2^2)^{1/2}}$$

The penetration depth is defined as the distance at which the intensity of the evanescent field has decayed to 1/e of the intensity at the reflection boundary. Typically, the evanescent wave propagates into a thin zone beyond the surface of a fiber with a penetration depth ranging from about 200 nm to 400 nm for visible light.

Fluorophores within the evanescent wave propagation zone are excited by that evanescent wave to emit fluorescence. Fluorophores further from the interface with the optical fiber will experience lower intensity of light at the excitation frequency and a resultant concomitant decrease in intensity of emitted fluorescence.

If the refractive index of the immobilized layer is substantially the same as the index of refraction of the substrate for immobilization (e.g., the glass surface of the optical element) the boundary for TIR effectively becomes the interface between the immobilized layer and the solution. Fluorophores bound to double-stranded nucleic acid in the immobilized layer experience direct excitation and, as a consequence, emit increased intensity fluorescence. For example, the index of refraction of a monolayer of organic media ($n_{monolayer}$ = 1.46 to 1.5; Ducharme et al., 1990, J. Phys. Chem. 94: 1925) is very similar to that of quartz (n_{quartz} = 1.46; O'Hanian, H.C. 1985, Physics, W. W. Norton & Co. N.Y. p. 835).

The use of surface immobilization methods, as described herein, which can generate an immobilized layer having a refractive index substantially equal to that of the optical element surface in the biosensors of this invention, result in

increased sensitivity and lower target nucleic acid detection limits.

Substantial matching of refractive indices of the optical element surface and the immobilized layer (i.e. substantial matching means that indices of refraction of the immobilized layer is within about ±10% of that of the surface) also results in increased sensitivity and lower detection limits for use of longer probe nucleic acids, particularly those equal to or longer than about 120 nucleic acid bases.

Light emitted from fluorophores (after evanescent or direct excitation) at the surface of the fiber is preferentially coupled back into the fiber and can be monitored by a photomultiplier tube (PMT) which is placed at an end of the fiber (Krull et al., 1991, Fiber Optic Chemical Sensors and Biosensors, Vol. II, O.S. Wolfbeis, Ed., CRC Press, Boca Raton, pp. 315). Increasing the length of coated fiber results in a greater optical path length and better sensitivity. Either evanescent or direct excitation of fluorophores in an immobilized layer extending from the biosensor results in improved signal to worse ratio as interference from background fluorescence in the bulk environment are avoided.

In the present invention, single-stranded nucleic acid sequences are covalently attached to the surface of the optical fiber. In a preferred embodiment, an automated DNA synthesizer is used to grow nucleotide oligomers onto the surface of activated optical fibers via the well established β-cyanoethylphosphoramidite method. Any commercially available automated DNA synthesizer can be used. The use of a "gene machine" to grow nucleic acids or nucleic acid analogs on the optical fiber substrates provides many advantages over conventional techniques of DNA immobilization. Conventionally, nucleic acid strands are adsorbed onto a suitable support (usually nitrocellulose) with little known about strand orientation. The use of a gene machine provides full control of

the oligomer sequence, strand orientation, and packing density in association with activation of the optical fiber substrates. Control over these parameters is critical to the development of a nucleic acid detection method based on hybridization as the alignment of the immobilized strands with respect to the availability of target nucleotides for hybridization and intermolecular interactions (electrostatic and steric) between oligomers will have direct ramifications on the kinetics of duplex formation and dissociation. The use of a gene machine, in addition to the chemistry used to activate the surface of the optical fibers, allows for the creation of membranes of desired density and structural order to permit rapid and reversible hybridization.

The optical fiber may be activated with a number of different compounds. The method of Arnold and co-workers (Arnold et al., 1989, Collect. Czech. Chem. Commun., 54: 523) may be used for the activation of the quartz wafers, optical waveguides, and optical fibers whereby 25 atom-long spacer molecules terminated by a dimethyoxytrityl protected nucleoside are immobilized onto the cleaned optical fiber substrate. In this method, the length of the spacer between the substrate and the first nucleoside is sufficiently long so that the environment of the terminal nucleoside is fluid enough to permit efficient coupling with successive nucleotide monomers during automated phosphoramidite synthesis. This is in accord with the report of Beaucage et al. (1992, Tetrahedron, 48: 2223-2311) wherein it was stated that nucleotide supports of lengths of at least 25 atoms are required to achieve high ($\geq$ 99.5%) coupling yields. The synthetic scheme requires inexpensive chemicals, is facile to perform, and is done as a one pot procedure wherein product isolation and purification is obviated. Because the linker is terminated by a protected nucleoside, any reactive sites on the support which would lead to the production of unwanted side products during automated synthesis can be eliminated by treating the derivatized supports with an acetic anhydride solution prior to synthesis. Lastly, the coverage of linker on the support is easily determined by

determining the amount of trityl cation released during the first trichloroacetic (TCA) deprotection step of the automated synthesis.

The use of the phosphoramidite method of oligonucleotide synthesis has been widely reviewed and has become the synthetic method of choice owing to the high coupling efficiencies and robustness of the reagents, in addition to the fact that the necessity of numerous product isolation and purification steps (which are required for liquid phase methods) are avoided. There are two readily available types of phosphoramidites which may be used to synthetically grow oligonucleotides, namely, methyl phosphoramidites and β-cyanoethyl phosphoramidites. The method utilizing β-cyanoethyl phosphoramidite is preferable as complete deprotection of the oligonucleotides can be done using aqueous ammonia (as opposed to thiophenol) for the case where oligonucleotides were grown onto controlled pore glass (CPG). Triethylamine is used to deprotect the β-cyanoethyl protected oligonucleotides grown onto quartz wafers or optical fibers without liberating the oligonucleotides from the support. An overview of the β-cyanoethyl phosphoramidite synthesis is as follows:

The first step in each cycle of solid phase automated phosphoramidite synthesis involves the removal of the dimethoxytrityl protecting group on the immobilized nucleotide. Detritylation is done by introducing a solution of 3% trichloroacetic acid (TCA) in 1,2 dichloroethane (DCE) onto the synthesis column in order to yield a 5′-hydroxyl functionality onto which the next nucleotide monomer may be coupled. TCA is the reagent of choice for detritylation due to its rapid reaction rate so that the oligonucleotide is only exposed to the acid for short periods of time, thereby avoiding the acid catalyzed removal of the adenine and guanine moieties from the nucleotide sugar groups by the process of depurination. Once the reaction has been completed, the acid is removed by flushing the column with acetonitrile. The eluent containing the released trityl

cation is sent to a fraction collector so that the coupling efficiency of the synthesis may be monitored by absorption spectroscopy.

Coupling is the next stage of the synthesis cycle. The contents of the synthesis column are dried by alternatively washing with acetonitrile and flushing with dry argon. This ensures that the support is anhydrous and free of nucleophiles. The desired phosphoramidite and tetrazole are then sent into the synthesis column. Tetrazole is a weak acid ($pK_a = 4.8$) which is used to activate the phosphoramidite. Nucleophilic attack by the 5'-hydroxyl group on the activated phosphoramidite moiety forms an internucleotide linkage. A ten-fold molar excess of phosphoramidite in an excess of tetrazole is added to the synthesis column to ensure that high coupling yields are achieved.

The next step of the synthesis is the capping step. This is done to eliminate further growth of sequences onto which coupling did not occur. The failed sequences are rendered unreactive by introducing acetic anhydride in the presence of the activating agent DMAP in order to acetylate any remaining unprotected 5'-hydroxyl moieties.

Because the trivalent internucleotide phosphite moieties are labile to both acidic and basic conditions, a solution of aqueous iodine is added after flushing the capping reagents from the column. This is done in order to oxidize the trivalent internucleotide phosphite moieties to the more stable pentavalent phosphate moieties found in naturally occurring nucleic acids. This procedure is termed the oxidation step.

Following the oxidation step, one cycle of nucleotide addition is complete. The process may be repeated many times until oligonucleotides of desired length and base sequence have been constructed. After addition of the last nucleotide, a final

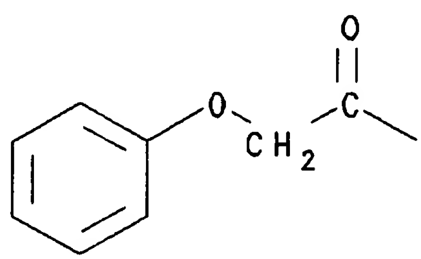
detritylation step is usually done in order to yield a 5'-hydroxyl group on the completed sequence.

Triethylamine is used for the removal of β-cyanoethyl protecting groups on the internucleotidic phosphotriester moieties of oligonucleotides grown onto optical substrates. This procedure is known to cause quantitative loss of the phosphate protecting groups via a β-elimination mechanism while not cleaving the single-stranded nucleic acids from the optical fibers. Ammonia treatment of the immobilized oligonucleotides is avoided by choosing an all-thymine base sequence. Thymine does not contain a primary amine functionality which would require protection during oligonucleotide synthesis. This approach is not limited to the use of phosphoramidite synthons, but is compatible with all commercially available solid-phase synthesis such as the H-phosphonate chemistry (Froehler, B.C., 1986, Tetrahedron Letters, 27: 5575; Stein et al., 1990, Analytical Biochemistry, 188: 11).

Contrary to the conventional preparation of oligonucleotides by solid-phase synthesis, post-synthesis removal of the product from the support is not desired. In order to prevent cleavage of the oligonucleotide from the support (optical fiber) while removing the protecting groups of the nucleobases, two modifications to the usual synthetic protocol can be made. The approach involves the combination of a hydrolysis resistant linkage between the oligomer and support along with the use of labile base protecting groups. Thus, an oligomer of any sequence can be prepared and deprotected yet remain attached to the support, available for hybridization.

The phenoxyacetyl (PAC) protecting group represents a convenient method for blocking the exocyclic amino functions of guanine, adenine and cytosine residues (thymine requires no nucleobase protection). The half-time of deprotection with concentrated ammonium hydroxide at 20°C is 8 min, 7 min and 2 min, respectively (Wu et al., 1989). Under these conditions, the

cyanoethyl phosphate protecting groups are removed within seconds (Letsinger and Ogilvie, 1969), whereas the linkage which joins the oligomer to the surface of the quartz fiber (e.g., a phosphodiester or phosphoramidate) is completely stable under these conditions. Alternative labile protecting groups are derivatized phenoxyacetyl groups including alkyl substituted PAC groups, more specifically t-butyl phenoxyacetyl groups. The t-butyl-phenoxyacetyl group can be quickly removed compared to hydrolysis of the linkage to the spacer thereby reducing the possibility of cleavage of the immobilized sequence from the surface. N-phenoxyacetyl deoxynucleoside 3'-cyanoethylphosphoramidites and the analogous t-butylphenoxyacetyl phosphoramidites are commercially available.

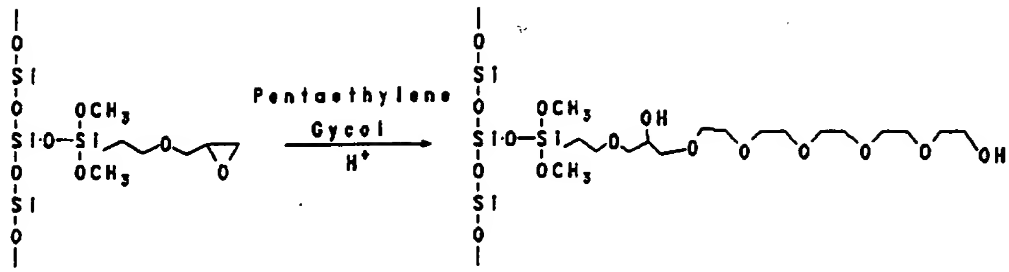


Other possible labile protecting groups could include the "FOD" (fast oligonucleotide deprotection available from Applied Biosystems Inc.) based on N,N-dialkylformamidines (Vinayak et al., 1992, Nucleic Acids Research, 20: 1265-1269). Use of protecting groups that can be selectively removed under conditions that will not cleave the oligomer from the support, such as the levulinyl group (removed by hydrazine treatment) (Letsinger et al., 1968, Tetrahedron Letters, 22: 2621-2624; Hassner et al., 1975, J. Amer. Chem. Soc., 97: 1614-1615) are also contemplated by the present invention. Even synthesis without nucleobase protecting groups is possible for up to 20-mers using the phosphoramidite approach (Gryaznov et al., 1991,

J. Amer. Chem. Soc., 113: 5876) or H-phosphonate chemistry (Kung et al., 1992, Tetrahedron Letters, 33: 5869). Either of these approaches circumvents difficulties in removing nucleobase protecting groups while leaving the oligomer attached to the support.

In conjunction with the above-described labile protecting groups, two types of hydrolysis resistant linkages may be used. Analogous to the natural internucleotidic linkage, a phosphodiester linkage is completely resistant to ammonolysis under the conditions which remove base-protecting groups. This linkage is produced by derivatization of optical fibers with the bifunctional silylating reagent 3-glycidoxypropyltrimethoxy silane. This yields a substrate derivatized with short spacer molecules terminated with an epoxide functionality. The length of the spacer arm is then extended by nucleophilic attack of pentaethylene glycol (PEG) in an acid catalyzed expoxide ring-opening reaction, yielding a stable ether linkage (Maskos et al., 1992 Nucl. Acids Res., 20(7) 1679). By extending the spacer molecule to one composed of at least 25 atoms, optimal coupling efficiencies are realized (Beaucage et al., 1992 Tetrahedron, 1992 48, 2223). This support, terminated with a hydroxyl functionality, is then used directly for automated oligonucleotide synthesis, obviating the need for tedious nucleotide functionalization of the support.

Since PEG is bifunctional, there exists the possibility of creating non-reactive closed-loop structures which may significantly decrease the amount of loading of oligonucleotides on the surface of an optical fiber. To eliminate any such problem, one terminus of PEG is protected with a dimethoxytrityl functionality prior to extensions of the glycidoxypropyltrimethyl silane. A second advantage provided by this strategy is facile determination of the amount of support linkers by monitoring Absorbance at 504nm (A_{504nm}) of the deprotection solution.



An amine-terminated solid support suitable for automated oligonucleotide synthesis may be prepared according to the method of Brennen et al. (1993, Sensors and Actuators B, 11: 109). A bifunctional amphiphilic support derivatization agent is created by condensing γ-aminopropyltriethoxysilane (APTES) with 12-nitrododecanoic acid. The resulting long chain spacer molecule is covalently immobilized onto the surface of the optical fibers by an S_n2 reaction between the hydroxyl groups present at the surface of the fiber and the silane moiety of the amphiphile. The support may then be capped using standard methods employed during automated synthesis (acetic anhydride), thereby masking other sites of reaction which may produce unwanted side products during oligonucleotide synthesis. Reduction of the terminal nitro-functionalities is then achieved by treatment of the derivatized support with an acidic zinc solution. The resulting amine headgroups may then be used directly for automated synthesis wherein an ammonolysis resistant phosphoramidate linkage is made between the activated support and the first nucleotide. An outline of a synthetic procedure used to immobilize alkyl amine monolayers covalently onto quartz substrates is depicted in Scheme I.

Free short strands of nucleic acids can also be covalently attached to the optical fiber directly or via linker molecules. This approach allows the use of DNA or RNA isolated from natural sources, amplified nucleic acids or their analogs, or synthetic samples provided in the fully deprotected form. Protocols provide end-attached oligomers of a well defined

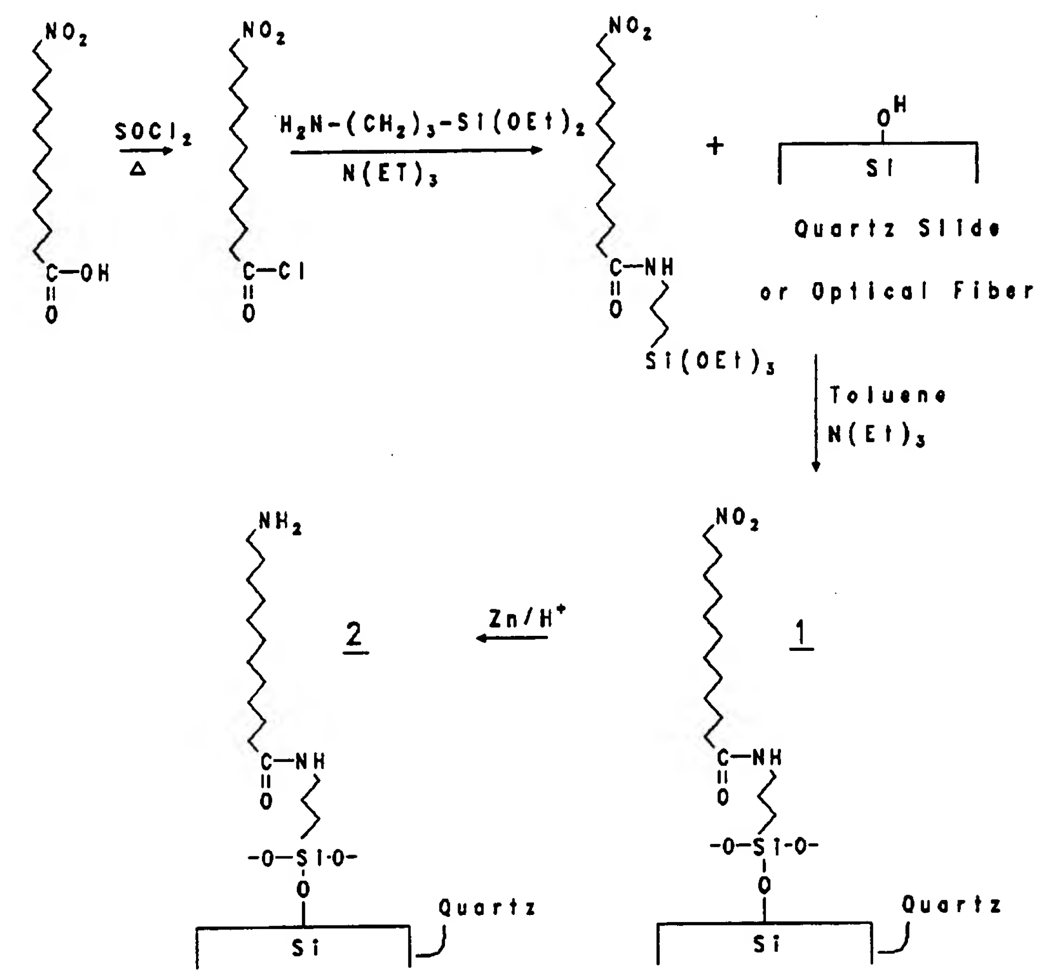
orientation. Hydrolysis-resistant linkages between the support and oligonucleotide may be employed to enhance the robustness of the biosensor.

Quartz optical fibers derivatized with linker molecules terminated with either hydroxyl or amino groups can serve as substrates for carbodiimide-mediated coupling with terminally phosphorylated single-stranded nucleic acids. Coupling to the hydroxyl fiber produces a phosphodiester bond while coupling to an amine fiber yields a phosphoramidate bond. Oligonucleotides can be phosphorylated, in solution, either chemically via a modification of Ouchi's method (Sowa et al. Bull. Chem. Soc., Japan 1975, 48 2084) or enzymatically.

Covalent attachment of free short strands of single-stranded nucleic acid to the optical fibers can be achieved by a slight modification of the method Ghosh and Musso (Ghosh and Musso, 1987, Nucl. Acids Res. 15: 5353). Coupling of a 5'-aminohexyl derivatized oligomer with activated carboxyl fibers affords end-attached oligomers. This method is known to minimize reaction at the amino groups of the DNA bases (which would potentially compromise the hybridization event) and affords surfaces with excellent nucleic acid coverage. The synthesis of the 5'- or 3'-terminally modified oligomers can be achieved readily by standard methods (Ghosh and Musso, 1987; Beaucage and Iyer, 1993).

Quartz optical fibers may also be activated by reacting 3-glycidoxypropyltrimethoxysilane with the hydroxylated fiber surface to form a silyl-ether linkage. The immobilized spacer is extended to a suitable length for DNA synthesis of oligonucleotide coupling by reacting pentaethylene glycol with the terminal epoxide moiety of the 3-glycidoxypropyl-trimethoxysilane. This provides an activated surface consisting of an oxygen rich long-chain spacer arm with a hydroxyl functionalized terminus onto which pre-purified oligonucleotide probes may be immobilized or oligonucleotides of desired base

SCHEME I


NO2
SOCl2
Δ
C—OH
O
C—Cl
H2N-(CH2)3-Si(OEt)2
N(ET)3
C—NH
Si(OEt)3
+
OH
Si
Quartz Slide
or Optical Fiber
Toluene
N(Et)3
NH2
2
Zn/H+
1
-O—Si-O-
Quartz

composition and sequence may be grown via solid phase phosphoramidite synthesis. This support has been shown to be stable to standard base and backbone deprotection conditions for the case where the oligonucleotide was chemically grown onto the support (Maskos et al., 1992, Nucl. Acids. Res., 20(7): 1679) and is sufficiently laden with ether functionalities so as to prevent the spacer arm from folding onto itself to prevent oligonucleotide coupling reactions from occurring.

The fluorophore or reporter group may be attached to the 5′- or 3′-end of the oligomer by not only a hydrocarbon tether but other types of tethers such as polyether, mixed aliphatic/aromatic, or peptidic. The tether need not be restricted to the 3′ or 5′ ends of the oligomer but may be attached to a terminal or internal ribo-residue via the 2′-hydroxyl (Yamana et al., 1991, Tetrahedron Letters, 32: 6347). Similarly, a tether can be attached to a terminal or internal nucleobase using pyrimidines (Pieles et al., 1990, Nucleic Acids Research, 18: 4355) or purines (Roduit et al., 1987, Nucleosides and Nucleotides, 6: 349). Furthermore, the internucleotidic linkage can be a site for tether attachment (Agrawal et al., 1990, Nucleic Acids Research, 18: 5419). Obviously, any combination of these methods could be used to site specifically incorporate multiple reporter groups.

The choice of fluorophores which may be tethered to the oligonucleotide include organic intercalating complexes, such as the commonly used nucleic acid stain ethidium bromide, thiozole orange and analogs thereof as prepared by L.G. Lee et al. (1986, Cytometry 7: 508) and the YOYO, BOBO, and TOTO series of fluorophores which are commercially available from Molecular Probes Inc. (Eugene, OR). Inorganic coordination complexes, such as the "molecular light switch" $Ru(phen')_2$ dppz PF_6 developed by Jenkins et al. (1992, J. Amer. Chem. Soc. 114: 8736) may also be used as well as groove binding dyes, such as Hoechst 33258 and Hoechst 33342, which are commercially available from Aldrich Chemical Co. (Milwaukee, WI). These fluorophores are chosen such

that little fluorescence is observed from the fluorophore when in the presence of single-stranded oligomers while intense luminescence is observed when in the presence of double stranded oligomers.

In one embodiment of the invention the biosensor operates as follows. The optical fiber with attached fluorescently labelled single-stranded nucleic acid is placed in a flow through cell and immersed in hybridization buffer solution. When single-stranded nucleic acids or nucleic acid analogs which are complementary to the immobilized strands are introduced to the flow cell, hybridization occurs followed by intercalation and enhanced fluorescence emission of the attached fluorescent probe. Fluorescence intensity is monitored in a total internal reflection configuration wherein the optical fiber is used as a waveguide to provide excitation to the surface immobilized nucleic acid and fluorescent probe, as well as to collect fluorescence emission. By monitoring the fluorescence intensity from the fiber, a measure of the amount of target nucleic acid in solution can be determined.

One instrument used for fluorescence intensity measurements is based on a fluorescence microscope as described elsewhere (Brennan et al., 1990, Anal, Chim. Acta., 237: 253). In alternative embodiments, the radiation source can be a frequency doubled laser, a semiconductor laser, bright lamp or LED. Coupling into the waveguide can be accomplished with fiber couplers, and the detector can be an avalanche diode rather than a PMT.

In another embodiment of the invention, the optical fiber with attached single-stranded nucleic acids is placed in a flow-through cell and immersed in hybridization buffer. When single-stranded nucleic acid or nucleic acid analogs which are complementary to the immobilized strands are introduced to the flow cell along with an environmentally sensitive fluorophore, hybridization occurs followed by intercalation of the fluorophore

and enhanced fluorescence emission of the attached fluorescent probe. Fluorescence intensity is monitored in a total internal reflection configuration wherein the optical fiber is used as a waveguide to provide evanescent excitation to the surface immobilized nucleic acid. By monitoring the fluorescence intensity from the fiber, a measure of the amount of target nucleic acid in the solution can be determined.

Regeneration of the biosensor can be achieved by thermal methods such as by rinsing the fiber in the flow-through cell with hot water or by chaotropic methods by rinsing the fiber with a hot salt solution. In either case, the duplex stability is reduced to the point where hybridization is not energetically favorable and the complement strands are dissociated from the covalently immobilized oligomers and flushed out of the flow cell. Regeneration methods as described herein can be employed to recycle biosensors.

The present invention is further illustrated by the following specific examples which are not intended in any way to limit the scope of the invention.

Example 1.

Preparation of Quartz Optical Fibers Derivatized with Long Chain Aliphatic Spacer Molecules Terminated with a 5'-O-dimethoxytrityl-2'-deoxythymidine Nucleoside

Plastic-clad silica optical fibers with a diameter of 400 μm were purchased from Tasso Inc. (Montreal, PQ, Canada). The cladding on the fibers was mechanically removed, and the fibers were cut to lengths of about 1 cm. One face on each fiber was polished by suspending the fiber over (and placing the end face of the fiber in contact with) the rotating plate of a Thermolyne type 37600 speed controlled mixer (Sybron Corporation, Dubuque, IO, USA) onto which 1200 grade emery paper was immobilized. All quartz optical fibers were cleaned using a Harrick PDG-32G plasma cleaner (Harrick Scientific Corporation, Ossining, NY, USA) before activation with aminopropyltriethoxy silane (APTES).

The fibers were then washed with a 1:1 acetone/methanol mixture and stored in a vacuum desiccator. The optical fibers were plasma cleaned for 5 minutes at low power (40 W) and were placed in a solution of 1:200 (v/v) aminopropyltriethoxy silane (APTES) in dry toluene. This was done under a nitrogen atmosphere using glassware which was previously treated with octadecyltrichlorosilane. The structure of the APTES coatings on quartz substrates has previously been investigated by Vandenberg et al. (1991, J. Colloid and Interface Sci., 147: 103). The method of Arnold and co-workers (1989, Collect. Czech. Chem. Commun., 54: 523) was used to synthesize an aliphatic spacer arm terminated with 5'-O-dimethoxytrityl-2'-deoxythymidine. In this method 1,10 decanediol was condensed with succinic anhydride to form 1,10 decanediol bis-succinate, as illustrated in Figure 3. The bis-succinate was reacted with N-hydroxysuccinimide and 5'-O-dimethoxytrityl-2'deoxythimidine in the presence of N,N'-dicyclohexyl-carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) to yield a nucleoside functionalized

spacer molecule. The spacer was then attached to the surface of the APTES treated optical fiber.

Example 2.

DNA Synthesis of Oligomers Onto Surface-Derivatized Quartz Optical Fibers

All DNA synthesis was done by the well established β-cyanoethylphosphoramidite method with an Applied Biosystems 381A DNA Synthesizer using controlled-pore glass beads, quartz optical fibers, or planar quartz wafers. Dimethoxytrityl cation released from each deprotection step of the oligonucleotide synthesis was quantitatively measured by absorption spectroscopy at 504 nm using a Hewlett Packard 8452A Diode Array Spectrometer (Hewlett Packard Corp., Palo Alto, CA, USA) to determine the percent coverage of dT_{20} on the optical fibers.

Automated solid-phase DNA synthesis is well known and is described in detail elsewhere (Beaucage et al., 1992, Tetrahedron Letters, 48: 2223-2311; Oligonucleotides and Analogues: A Practical Approach, F. Eckstein, Ed. Oxford University Press, NY, 1991). The surface-derivatized optical fibers were placed into an emptied Applied Biosystems (ABI) Oligonucleotide Purification Cartridge column (OPC-column) with the dead volume being taken up by inert packing material. The end filter papers were replaced (ABI) and the column ends were crimped closed using aluminum seals. Synthesis of oligomers onto the optical fibers was carried out at the 0.2 μmol. scale with a pulsed-delivery cycle in the "trityl off" mode. The β-cyanoethylphosphoramidite cycle was used as supplied by ABI with the exception of extended nucleoside coupling times (2 min.). Deprotection of the phosphate blocking groups from the immobilized oligomer was achieved by standing the fibers in a solution of 2:3 triethylamine/acetonitrile at room temperature for 1.5 hours. This procedure caused the loss of the phosphate

blocking group via a β-elimination mechanism while not cleaving the single-stranded DNA (ssDNA) from the optical fibers.

Fibers coated in ssDNA were either stored under vacuum or kept in a solution of 1:1 ethanol/water. Fibers stored under vacuum were cleaned prior to use by sonication in a solution of 1:1 ethanol/water for 5 minutes in order to remove any fluorescent contaminants adsorbed to the surface of the fibers.

Example 3.

Biosensor Characterization by Trityl Cation Assay

All oligonucleotide syntheses were evaluated by spectroscopic quantitation of trityl cation released during the trichloroacetic acid treatment steps of the automated synthesis. The collected fractions of trityl cation were diluted with 2.0 mL of 5% TCA in 1,2-dichloroethane immediately prior to making absorbance measurements. Absorption at 504 nm was measured in order to determine the concentration and the total number of trityl cations released during each deprotection step of the synthesis. In this way, the total number of oligomers successfully grown onto the solid supports was determined.

As there exists no discernible decrease in the amount of trityl cation released during successive deprotection steps, it may be safely assumed that the coupling efficiency of 99.5% or better suggested by the manufacturer of the automated synthesizer (ABI) was achieved. The coverage of the quartz substrates with oligonucleotides by methods of examples 1 and 2 was then calculated as follows.

Assuming that the quartz surfaces were perfectly flat, the total surface area of all the quartz substrates placed into the synthesis column was calculated to be $1.1 \pm 0.1 \times 10^{15}$ nm^2. On average 8.8 ± 0.1 nanomoles of dimethoxytrityl cation were released for each deprotection step. Given that the amount of

dimethoxytrityl released by the final detritylation step is equal to the number of DNA strands successfully grown, a loading of 5 molecules of dT_{20} per square nanometer of quartz was calculated. However, the area on the surface occupied by each strand of immobilized DNA was calculated to be 3 nm^2 based on the diameter of the helical structure.

Example 4.

Generation of Complementary and Non-complementary Nucleic Acids

Synthesis of dA_{20} and rA_{20} was done using a conventional LCAA-CPG support with the β-cyanoethylphosphoramidite cycle supplied by ABI. A nonadecamer of random base composition (dR_{19}) was also prepared by simultaneously introducing all four phosphoramidite reagents to the column at each coupling step. Standard deprotection with aqueous ammonia (29%, 1.5 mL, 24 h) was used to liberate the oligomers from the solid support and remove the base protecting groups. For the case of the rA_{20}, deprotection of the phosphate blocking groups, base protecting groups and cleavage from the CPG support was done by treating the oligomers with 1.5 mL of a solution consisting of 4 parts aqueous ammonia and 1 part ethanol for 48 hours at room temperature. The aqueous solution containing the oligonucleotides was then collected, evaporated to dryness, and the residue treated with 300 μL of an anhydrous solution of 1 M tetra-N-butyl ammonium fluoride in THF overnight at room temperature. After the incubation time, the reaction was quenched by adding 1 mL of water to the reaction mixture. Crude oligomer was purified by polyacrylamide gel electrophoresis and reverse phase liquid chromatography or size exclusion chromatography.

Example 5.

Detection and Quantification of cDNA by the Optical Sensor of Example 2

The instrument used for fluorescence intensity measurements is based on a fluorescence microscope as described elsewhere (Brennan et al., 1990, Anal, Chim. Acta., 237: 253).

A DNA coated fiber was selected at random from the batch of fibers (ca. 25) onto which ssDNA was grown and was positioned under the objective of the microscope. In this orientation the incident laser radiation entered the fiber at one end and was totally internally reflected. The majority of the fiber was submerged in a hybridization buffer solution consisting of 0.9 M NaCl and 50 mM sodium phosphate (pH 7.4) in sterile water. Hybridization buffer was passed through an acrodisc filter immediately prior to introduction to the cuvette.

100 μL of a 2.75 $\mu g \cdot mL^{-1}$ or various volumes of a 56.8 $\mu g \cdot Ml^{-1}$ aqueous solution of purified dA_{20} ssdna was added to the plastic cuvette containing the suspended fiber in fresh hybridization buffer at 85°C. The solution was allowed to stand and cool to room temperature (25°C) between 30 and 90 minutes after which the fiber was flushed with 60 Ml of hybridization buffer (25°C).

Intercalation of the fluorophore into the dsDNA was achieved by injecting 10 μL of a 1 $mg \cdot ml^{-1}$ aqueous solution of ethidium bromide (EB) into the cuvette and allowing the solution to stand for 15 minutes followed by washing the fiber by flushing the cuvette with 60 Ml of fresh hybridization buffer (25°C).

The instrument used for fluorescence intensity measurements is illustrated in Figure 2, and was based on an inverted microscope as designed by Krull and co-workers [Brennan

et al. Anal. Chim. Acta. 1990, 237, 253.]. Laser radiation was focused into the fiber optic biosensor. Emitted fluorescent radiation, from the stimulated fluorescent molecules associated with the double-stranded nucleic acids, was directed back towards the microscope by total internal reflection. The emission from the fluorescent molecules was separated from the excitation radiation by a dichroic mirror and directed to a photomultiplier tube. The photomultiplier tube provided measurements of the intensity of fluorescence emission (see Figures 4 and 5).

Fluorescence intensity values are reported as relative quantities obviating the need to control experimental parameters such as laser intensity, optical alignment and photomultiplier tube (PMT) gain which are beyond accurate control from day to day.

The response of the fiber optic DNA biosensor to EB and CDNA is shown in Figure 4. The optical fiber coated with immobilized ssdna was taken from storage under vacuum, was placed under the fluorescence microscope, and the initial fluorescence intensity with the fiber submerged in the hybridization buffer (25°C) was measured. After two hours the fluorescence intensity from the fiber was measured again, and the results indicated that there was no appreciable drift in the response of the instrument or the amount of fluorescent material present at the surface of the fiber (Figure 4, section A).

Fluorescence microscopy studies of the surface of fibers which were stored under vacuum indicated the presence of some fluorescent contaminants. The contribution of these fluorescent contaminants was responsible for much of the fluorescence intensity observed in Figure 4, section A. As a control experiment, 10 μL of a 1 mg·ml^{-1} aqueous solution of EB was added to the cuvette (3 Ml) in which the fiber was suspended. After 15 minutes, 60 Ml of fresh hybridization buffer (25°C) was flushed through the cuvette in order to remove any non-specifically bound ethidium cation. A decrease in the

fluorescence intensity back to baseline values was observed after this washing, as shown in Figure 4, section B, due to the removal of fluorescent contaminants from the surface of the optical fiber.

The room-temperature hybridization buffer present in the cuvette was replaced with hot (85°C) hybridization buffer and 257 ng of complementary (dA_{20}) ssDNA was then injected into the cuvette and the system allowed to cool for 90 minutes. This temperature was chosen as it is sufficiently greater than the 60°C duplex melting temperature (T_m, the temperature at which half of all the duplexes present are dissociated) and is well below the boiling point of the buffer. Incubation at temperatures below T_m has been shown to cause incomplete hybridization wherein only a fraction of the bases on each strand interact to form partially hybridized complexes (Rubin et al., 1989, Nucleic Acid and Monoclonal Antibody Probes, B. Swaminathan and G. Prakash, Eds., Marcel Dekker, Inc., NY, pp. 185-219). Though covalent immobilization of ssDNA removes one degree of freedom from the oligomer, hybridization at temperatures initially above the duplex T_m ensures the formation of duplexes with the greatest possible extent of overlap. No appreciable intensity change from that of the baseline was observed after the 90 minute incubation period (Figure 4, section C). 10 µL of the EB solution was added to the buffer, and the solution was allowed to stand for a 15 minute incubation time. The fiber was then washed with 60 mL of fresh buffer as described for the blank experiment. A 50 ± 20% increase in the fluorescence intensity as shown in Figure 4, section D was observed from the fiber which was coated in EB labeled dsDNA. It is interesting to note that 257 ng (4.11 femtomoles) of dA_{20} only amounts to 2 x 10^{-5}% of the total available ssDNA immobilized onto the fiber. In order to ensure that the ethidium cation that was present was intercalated into the dsDNA, the fiber was washed with an additional 30 mL of buffer solution. After this washing, no appreciable decrease in the fluorescence intensity was observed (Figure 4, section D).

Example 6.

Response of the Sensor of Example 2 to Complement RNA (cRNA)

A 3.8ng·μl^{-1} solution of rA_{20} (450μl) was introduced into a cuvette containing hot hybridization buffer giving a 570ng·ml^{-1} solution of cRNA. A hybridization and staining procedure, the same as that used for DNA-DNA hybridization on the sensor surface, was followed. A comparison of response of the biosensor with immobilized dT_{20} to cDNA and cRNA agree to within experimental error. In contrast, a similar concentration of non-complementary sequences gave essentially no response.

Example 7.

Effective of Ethidium Bromide (EB) Staining Time and Concentration

The staining time of the sensor with EB was changed after each hybridization with cDNA. For each determination, injections of 30μl of 56.8μg·ml^{-1} solution of aqueous dA_{20} were made and the hot hybridization buffer in the cuvette, which contained the cDNA strands, was allowed to cool to room temperature over a time of 30 minutes. A 1 mg·ml^{-1} solution of EB in water (10μl) was added to the cuvette after each hybridization to provide an EB concentration of 8.4×10^{-3}M. A staining time of 20 min. with 8.4×10^{-3}M EB was required to generate $\geq$99% of the full signal.

To study the effect of EB concentration during dsDNA staining, all hybridization parameters were the same as those used to study staining time and a staining time of 20 min. was used. Staining with EB solutions of concentrations of 8.5×10^{-4}M or greater were required to generate $\geq$99% of the full staining in 20 min.

Example 8.

Regeneration of the Nucleic Acid Sensor

To determine if the nucleic acid sensor could be regenerated, the fiber was washed with 30 mL of hot (85°C) buffer solution over a period of about thirty seconds and the system allowed to incubate in the hot buffer for five minutes. After the five minute wait, an additional 30 mL of hot buffer was flushed through the cuvette to wash away the dissociated cDNA strands. This procedure is known to melt DNA duplexes, as the buffer temperature was well above the T_m of the dsDNA. The fluorescence intensity returned, within experimental uncertainty, to the initial intensity observed at the beginning of the experiment (Figure 4, section E). As a control experiment, EB was introduced into the cuvette and was then washed out as done previously. The fluorescence intensity remained at the initial value, as shown in Figure 4, section F, indicating that the DNA duplexes had indeed been dissociated and the complement strands were removed.

To test the reproducibility of the sensor, another 257 ng of cDNA was added to the cuvette and after 90 minutes the fiber was treated with EB, was then washed, and the fluorescence intensity was again measured. An increase in fluorescence intensity was observed which was similar in magnitude to that observed from the first dsDNA analysis (Figure 4, section G). After washing the fiber with hot buffer solution, a baseline intensity within the experimental uncertainty of the results was again observed (Figure 4, section H).

A calibration experiment was done to test the analytical response of the DNA biosensor. The procedures used in this experiment were identical to that of the first experiment with the exception that 30 minute incubation times for hybridization were used as it was found that only 30 minutes was required for the 85°C buffer that was added to the cuvette to

cool to 25°C. Good reversibility of the signal was observed (Figure 5) as well as a signal which was linear with the amount of cDNA added to the cuvette (Figure 6). The regression line shown in Figure 6 shows a good fit to the data points with an r^2 value of 0.965. From these data, the sensitivity of the sensor was determined to be an increase in fluorescence intensity of 83% per 100 $ng \cdot mL^{-1}$ of cDNA with a measured limit of detection of 86 $ng \cdot mL^{-1}$.

The robustness of the fibers, and DNA as a biorecognition element, was made evident by the maintenance of activity after long-term storage and stringent cleaning conditions. Fibers that were stored for up to eight months in vacuo or in 1:1 ethanol/water solutions possessed identical response characteristics to freshly prepared fibers. Adsorbed fluorescent contaminants which were accumulated through long-term storage were completely removed (as confirmed through fluorescence microscopy) by sonicating the fibers in a solution of 1:1 ethanol/water with full maintenance of activity and sensitivity. The response to 86 $ng \cdot mL^{-1}$ of cDNA was a 50 ± 20% increase in fluorescence intensity for a freshly prepared sensor (Figure 4, section D), which correlates well to the 104 ± 15% intensity increase observed when the 1 month old sensor was treated with 189 $ng \cdot mL^{-1}$ of cDNA (Figure 5, section D).

A control experiment was also done in which 30 μL of a 60 $\mu g \cdot mL^{-1}$ solution of 19 nucleotide long oligomer with random base composition was injected into the cuvette containing hot (85°C) buffer followed by incubation for 30 minutes. Washing of the fiber after incubation with dR_{19} and treatment with EB was done as described previously. No change in intensity was observed for this control experiment (Figure 5, sections H-J) indicating that the sensor was sequence specific.

Biosensors stored for 11 months, dry or in ethanol, were subjected to cleaning by sonication in absolute ethanol (15

min.) and autoclaving (120°C for 20 min. at 4 atm. pressure). The response of the biosensor to cDNA ~400 ng·ml^{-1} improved most likely through removal of contaminants on the sensor surface.

Example 9.

Immobilization and Deprotection of Mixed Base Sequence Nucleic Acids Without Cleaving the Oligonucleotides From the Support

In order to grow oligonucleotides onto the surface of silica substrates (such as quartz) by automated solid phase synthesis, the surface is functionalized with spacer molecules of at least 25 Å in length which had either an amine or a hydroxyl functionality at the terminus of the spacer molecule. A chemically resistant, non-hydrolyzable spacer molecule is employed. The method used was a modification of that reported by U. Maskos and E.M. Southern supra wherein the silica surface was treated with glycidoxypropyltrimethoxysilane (GOPS), followed by extension *via* treatment with pentaethylene glycol (PEG) under acidic conditions. For the purpose of creating biosensors with higher sensitivity and lower detection limits, this method is advantageous over the use of hydrocarbon tethers. The water soluble PEG linker will provide a more fluid environment (which should not self-assemble) so as to improve the ability of the immobilized DNA strands to hybridize with complementary material in solution (in terms of energetics and kinetics). The hydrophylicity of the linker will also facilitate the removal of adsorbed contaminants (e.g. proteins, organics) which may occlude the surface and contribute to drift in the fluorescence intensity. However, as PEG is bifunctional, there exists the possibility of creating non-reactive closed-loop structures which may significantly decrease the loading of oligonucleotides on the surface of the fibers. In order to eliminate this problem, one terminus of the PEG is protected with a dimethoxytrityl functionality prior to extension with GOPS. This strategy permits facile determination of the amount of support linkers bound to the silica surface. Removal of the trityl protecting

groups by treatment with acid yields the highly colored trityl cation, which can be quantitated by monitoring $A_{504(nm)}$ of the deprotection solution. Knowing there is one trityl group released per linker molecule attached to the surface, the loading of PEG can easily be determined. Immobilization of a protected linker molecule provides the additional advantage that the hydroxyl groups produced after the attachment of the PEG to the epoxide moiety and all other surface silanols can be capped to prevent unwanted oligonucleotide growth at these sites. The presence of side product oligonucleotides, which are prematurely terminated due to the lack of a suitable support molecule, may decrease the sensitivity and selectivity of the sensor. The additional charge imparted from the anionic backbone of a side-product strand may inhibit hybridization between the analyte strands and neighboring probe sequences. See: R.T. Pon Methods in Molecular Biology, Vol.20: Protocols for Oligonucleotides and Analogs, S. Agrawa, Ed, 1993, Humana Press, Inc. Totowa NJ. In conjunction with the use of non-hydrolyzable spacer molecules, t-butylphenoxyacetyl protected phosphoramidite synthons were employed. This labile protecting group can be quickly removed (15 min @ 55°C or 120 min. @ room temp as compared to 12-16 hours @ 55°C using 27% aqueous ammonia) thereby reducing the possibility of cleavage of the immobilized sequences by hydrolysis of the silyl ether bonds which ultimately anchor the strands to the fiber surface.

Cleaning of Silica Substrates Prior to Functionalization with GOPS: The quartz substrates, i.e., optical fibers or wafers, were added to a 1:1:5 (v/v) solution of 30% ammonium hydroxide/30% hydrogen peroxide/water and the mixture was stirred at 80°C for five minutes. The substrates were then removed and treated with a solution of 1:1:5 (v/v) conc. HCl/30% hydrogen peroxide/water and the mixture stirred at 80°C for five minutes. The substrates were then sequentially washed with methanol, chloroform and diethyl ether, respectively, and dried *in vacuo*.

Synthesis and Purification of monotritylated pentaethylene glycol (DMT-PEG): A solution of 7.1g dimethoxytrityl chloride in 10 ml of dried and distilled pyridine was added dropwise to a stirred solution of 5.65 ml of pentaethylene glycol and 5 ml of pyridine under an argon atmosphere. Stirring was continued overnight after which the reaction mixture was combined with 50 ml of dichloromethane. The mixture was then twice shaken with 900 ml of 5% aqueous bicarbonate and twice more with 900 ml of water to remove unreacted PEG, pyridine, and pyridinium salts. The product was purified by liquid chromatography using silica gel and a solvent system of 0.1% triethylamine in dichloromethane. The identity of the product was confirmed by proton NMR spectroscopy (200 MHz).

Functionalization of Silica Substrates with 3-Glycidoxypropyl Trimethoxy Silane (GOPS): The cleaned quartz substrates were suspended in a stirred solution composed of 40 ml xylene, 12 ml GOPS, and a trace of Hünig's base at 80°C overnight. The fibers were then sequentially washed with methanol, chloroform, ether, and then dried *in vacuo*.

Linkage of DMT-PEG to GOPS Functionalized Silica Substrates: The GOPS functionalized fibers were suspended in a stirred solution of 1:4:8 (v/v) DMT-PEG/diethyl ether/toluene containing a catalytic amount of sodium hydride under an argon atmosphere. The reaction mixture was stirred for 14 days after which time the fibers were removed and washed sequentially with methanol, chloroform, ether, and then dried *in vacuo*.

Capping of Unreacted Silanol and Hydroxyl Functionalities with Chlorotrimethyl Silane: The quartz fibers functionalized with DMT-PEG were suspended in a solution of 1:10 (v/v) chlorotrimethylsilane/pyridine overnight under an argon atmosphere at room temperature.

Synthesis of Oligomeric Probe for *Candida Albicans* onto PEG Functionalized Optical Fibers: For introduction onto the DNA synthesizer, the DMT-PEG functionalized optical fibers were placed into an empty 10-μmol scale column with the dead volume being taken up by inert packing material. The end filter papers were replaced and the column ends were crimped closed using aluminum seals. Synthesis of oligomers onto the optical fibers was done using the 0.2-μmol scale synthesis cycle supplied by the manufacturer (Applied Biosystems Inc. (ABI)) in the "trityl off" mode using extended nucleoside coupling times (5 min) and increased reagent delivery times (x5). t-Butylphenoxyacetyl protected phosphoramidite synthons were used in conjunction with a t-butylphenoxyacetic anhydride capping solution as supplied by Millipore Inc. Trityl cation released from each deprotection step was collected and quantitated by A_{504nm} in order to determine the loading of DMT-PEG on the fibers and to monitor the nucleotide coupling efficiency. The oligonucleotide sequence grown on the functionalized quartz substrates was (5'-TAG GTG AGA CAT ATC ACA GA-3').

Deprotection of Phosphate and Exocyclic Amine Blocking Groups: A 30% ammonium hydroxide solution was drawn up into the synthesis column containing the optical fibers functionalized with immobilized oligonucleotides using a syringe and a male-to-male luer adapter. The fibers submerged in ammonia were allowed to stand for two hours at room temperature after which time the ammonia solution was expelled from the column and the contents of the column were washed five times with 5 ml portions of sterile water. The deprotection solutions and washings were collected and concentrated to a total volume of 1 ml. A_{260nm} of the concentrated deprotection solution was measured in order to determine the quantity of DNA liberated from the quartz substrates. Based on the results of the trityl cation assay and A_{260nm} of the deprotection solution, it was found that 30% of the oligomers remained attached to the surface following the ammonia deprotection procedure.

A UV-Visible scan of the surface of a quartz wafer functionalized with covalently attached oligonucleotides (prepared with the optical fibers) shows a significant peak at 260nm, indicative of the presence of immobilized DNA. The oligonucleotide sequence grown on the functionalized quartz fibers (5′-TAG GTG AGA CAT ATC ACA GA-3′) is a nucleic acid probe for the EO^3 Forward sequence of the *Candida Albicans* genome.

Example 10

Synthesis and Deprotection of 5′-aminohexyl poly thymidilic acid icosanucleotides Immobilized on Quartz Optical Fibers Functionalized with DMT-PEG-GOPS Linker Molecules

DMT-PEG-GOPS functionalized optical fibers (prepared as in Example 9) were placed into an empty OPC-column (ABI) with the dead volume being taken up by inert packing material. The end filter papers were replaced and the column ends were crimped closed using aluminum seals. Synthesis of oligomers onto the optical fibers was done using the 0.2-μmol scale synthesis seals. Synthesis of oligomers onto the optical fibers was done using the 0.2-μmol scale synthesis cycle supplied by the manufacturer (ABI) in the "trityl off" mode using extended nucleoside coupling times (5 min). Deprotection of the phosphate blocking groups from the immobilized oligomers was achieved by standing the fibers in a solution of 2:3 (v/v) triethylamine/acetonitrile at room temperature for 1.5 hours. Removal of the trifluoroacetamide protecting group on the aminohexyl functionality located at the 5′-end of the immobilized strands was done by exposing the fibers to a 10^{-3} M solution of sodium borohydride in absolute ethanol for 1 hour at room temperature. The fibers were then washed once in a solution of 10^{-3} M HCl followed by washing with copious amounts of sterile water.

<u>Attachment of the Trityl-Protected Tethered Ethidium Analogue to the Aminohexyl Functionalized Optical Fibers</u>: The fully deprotected 5'-aminohexyl polythymidilic acid icosanucleotides immobilized on quartz optical fibers were suspended in a solution containing 5mg of the DMT-protected tethered ethidium analogue, 40μl of 1-methylimidazole, and 1.91 g of 1-ethyl-3-(3-dimethylaminopropylcarbodiimide in 50 ml of water. After a 7 day incubation period at room temperature, the fibers were washed five times each with water, ethanol, and dichloromethane respectively. The proportion dye-functionalized oligonucleotides was determined by quantitating the amount of trityl released from each detritylation step during automated synthesis and that from the deprotection procedure used to restore the primary amine moieties on the dye. From these assays it was determined that 63% of the immobilized oligonucleotides were functionalized with tethered dye.

<u>Characterization of the Fluorescence Response of the Reagentless Sensors with Tethered Fluorophore</u>: The response of the reagentless sensor to 720 ng of complement DNA is shown in Figure 9. Hybridization was done at 40°C in a buffer consisting of 1 M NaCl and 50 mM phosphate (pH 7.0). It should be noted that this sensor has a significantly improved response time over the sensors without tethered dye. 99% of the full analytical signal was reached in about 6 minutes after injection of the complementary strands for the reagentless system while 45 minutes was required for full signal generation by the sensors without tethered dye.

We claim:

1. A biosensor system for detecting a target single-stranded nucleic acid which comprises:

a light source;

an optical element for receiving light from said light source and conveying the light to an interaction surface of the optical element;

single-stranded nucleic acid, the sequence of which is, at least in part, complementary to the sequence of said target nucleic acid and which is immobilized onto said interaction surface of the optical element forming an immobilized layer on said surface;

fluorescent molecules capable of intercalating in double-stranded nucleic acid within said immobilized layer on said surface and which will emit fluorescence when stimulated by light from said light source;

a detector for emission of fluorescence from said intercalated fluorescent molecules wherein said emission of fluorescence detects hybridization of said immobilized single-stranded nucleic acid with said target nucleic acid and thereby detects said target nucleic acid.

2. A biosensor system according to claim 1 wherein said optical element comprises an optical waveguide having a substrate that confines light by total internal reflection.

3. A biosensor system according to any of claims 1 or 2 wherein said optical element comprises an optical fiber.

4. A biosensor system according to any of claims 1-3 wherein said optical element conveys at least a portion of the emitted fluorescence to said detector.

5. A biosensor according to any of claims 2-4 wherein the immobilized layer has a refractive index substantially the same as that of the substrate that confines light in said optical element.

6. A biosensor according to claim 5 wherein the immobilized single-stranded nucleic acid is equal to or greater than 120 bases in length.

7. A biosensor system according to any of claims 1-6 wherein said fluorescent molecules are in a solution in which the optical element is immersed.

8. A biosensor system according to any of claims 1-6 wherein said fluorescent molecules are bound to said immobilized single-stranded nucleic acid.

9. A biosensor system according to claim 8 for detecting a first and a second target nucleic acid each target nucleic acid having a distinct nucleic acid sequence wherein said single-stranded nucleic acid immobilized onto said interaction surface comprises a first and a second single-stranded nucleic acid having a sequence complementary to said first and second target nucleic acids, respectively, and wherein said fluorescent molecules bound to said immobilized nucleic acid comprise a first fluorescent molecule which emits fluorescence at a first wavelength bound to said first immobilized complementary nucleic acid and a second fluorescent molecule which emits fluorescence at a second wavelength bound to said second immobilized complementary nucleic acid.

10. A biosensor system according to any of claims 1-9 wherein said single-stranded nucleic acid is immobilized to said

interaction surface through a covalent bond to a linker molecule which in turn is covalently bonded to said surface.

11. A biosensor system according to claim 10 wherein said linker molecule is greater than or equal to about 25 Angstroms in length.

12. A biosensor system according to any of claims 10-11 wherein the linker results from initial surface reaction with glycidoxypropyltrimethyoxysilane followed by extension by treatment with pentaethylene glycol.

13. A biosensor system according to any of claims 1-12 wherein said single-stranded nucleic acid is immobilized to said interaction surface by *in situ* nucleic acid synthesis.

14. A biosensor system according to claim 13 wherein the linkage between the oligomer being synthesized and the substrate comprises a hydrolysis-resistant linkage.

15. A biosensor system according to any of claims 13-14 wherein a labile base protecting group is employed during *in situ* synthesis.

16. A biosensor system according to claim 15 wherein the labile base protecting group is a phenoxyacetyl group.

17. A biosensor system according to any of claims 1-16 which can detect a target nucleic acid at a concentration less than or equal to about 1 nanomole of a target nucleic acid.

18. A biosensor system according to any of claims 1-16 which can detect a target nucleic acid at a concentration less than or equal to about 10 femtomoles of a target nucleic acid.

19. A biosensor system according to any of claims 1-18 wherein at least the portion of said optical element comprising said interaction surface and said immobilized layer is disposable.

20. An optical fiber for use in detection of a target nucleic acid which comprises an interaction surface having single-stranded nucleic acid, the sequence of which is, at least in part, complementary to the sequence of said target nucleic acid immobilized upon said surface to form an immobilized layer.

21. The optical fiber of claim 20 wherein said single-stranded nucleic acid is immobilized to said interaction surface by in situ nucleic acid synthesis.

22. The optical fiber according to any of claims 20-21 further comprising fluorescent molecules capable of intercalating in double-stranded nucleic acid and which will emit fluorescence when stimulated by a light source wherein said fluorescent molecules are covalently bound to said immobilized single-stranded nucleic acid.

23. The optical fiber according to claim 22 for detecting a first and a second target nucleic acid each target nucleic acid having a distinct nucleic acid sequence wherein said single-stranded nucleic acid immobilized onto said interaction surface comprises a first and a second single-stranded nucleic acid having a sequence complementary to said first and second target nucleic acids, respectively, and wherein said fluorescent molecules bound to said immobilized nucleic acid comprise a first fluorescent molecule which emits fluorescence at a first wavelength bound to said first immobilized complementary nucleic acid and a second fluorescent molecule which emits fluorescence at a second wavelength bound to said second immobilized complementary nucleic acid.

24. The optical fiber according to any of claims 20-23 wherein the immobilized layer has a refractive index substantially the

same as that of the substrate that confines light in said optical element.

25. The optical according to claim 24 wherein the immobilized single-stranded nucleic acid is equal to or greater than 120 bases in length.

26. The optical fiber according to any of claims 20-25 wherein said single-stranded nucleic acid is immobilized to said interaction surface through a covalent bond to a linker molecule which in turn is covalently bonded to said surface.

27. The optical fiber according to any of claims 20-26 wherein the linkage between the oligomer being synthesized and the substrate comprises a hydrolysis-resistant linkage.

28. The optical fiber according to any of claims 20-27 wherein said linker molecule is greater than or equal to about 25 Angstroms in length.

29. The optical fiber according to any of claims 20-28 wherein the linker results from initial surface reaction with glycidoxypropyltrimethyoxysilane followed by extension by treatment with pentaethylene glycol.

30. The optical fiber according to any of claims 20-29 wherein said single-stranded nucleic acid is immobilized to said interaction surface by in situ nucleic acid synthesis.

31. The optical fiber according to claim 30 wherein a labile base protecting group is employed during in situ synthesis.

32. The optical fiber according to claim 31 wherein the labile base protecting group is a phenoxyacetyl group.

33. The optical fiber according to any of claims 20-32 which is disposable.

34. A method for detecting a target nucleic acid employing the biosensor system according to any of the claims 1-19.

35. A method for detecting a target nucleic acid employing the optical fiber according to any of the claims 20-33.

36. A method for detecting the presence of a pathogenic microorganism or virus in a sample which comprises

providing an optical element having an interaction surface with an immobilization layer on that surface which comprises single-stranded nucleic acid having a selected nucleic acid sequence that is at least in part complementary to a target nucleic acid sequence which is characteristic of and selective for said microorganism or said virus;

pretreatment of said sample such that target single-stranded nucleic acids characteristic of or selective for said microorganism or virus that may be present in said sample are capable of hybridizing to their complements;

contacting the interaction surface of said optical element with said pretreated sample such that said target nucleic acids can hybridize to said immobilized single-stranded nucleic acids;

providing fluorescent molecules in proximity to the interaction surface of the optical element such that the fluorescent molecules will intercalate upon hybridization of target nucleic acid with complementary immobilized single-stranded nucleic acid;

illuminating said intercalated fluorescent molecules with light such that fluorescence is stimulated; and

detecting the emitted fluorescence whereby the presence of said microorganism or virus in said sample is detected.

37. A method for detecting the presence of a pathogenic microorganism or virus by detecting the presence of a target nucleic acid that is characteristic of or selective for said microorganism or virus which comprises employing the biosensor system according to any of claims 1-19.

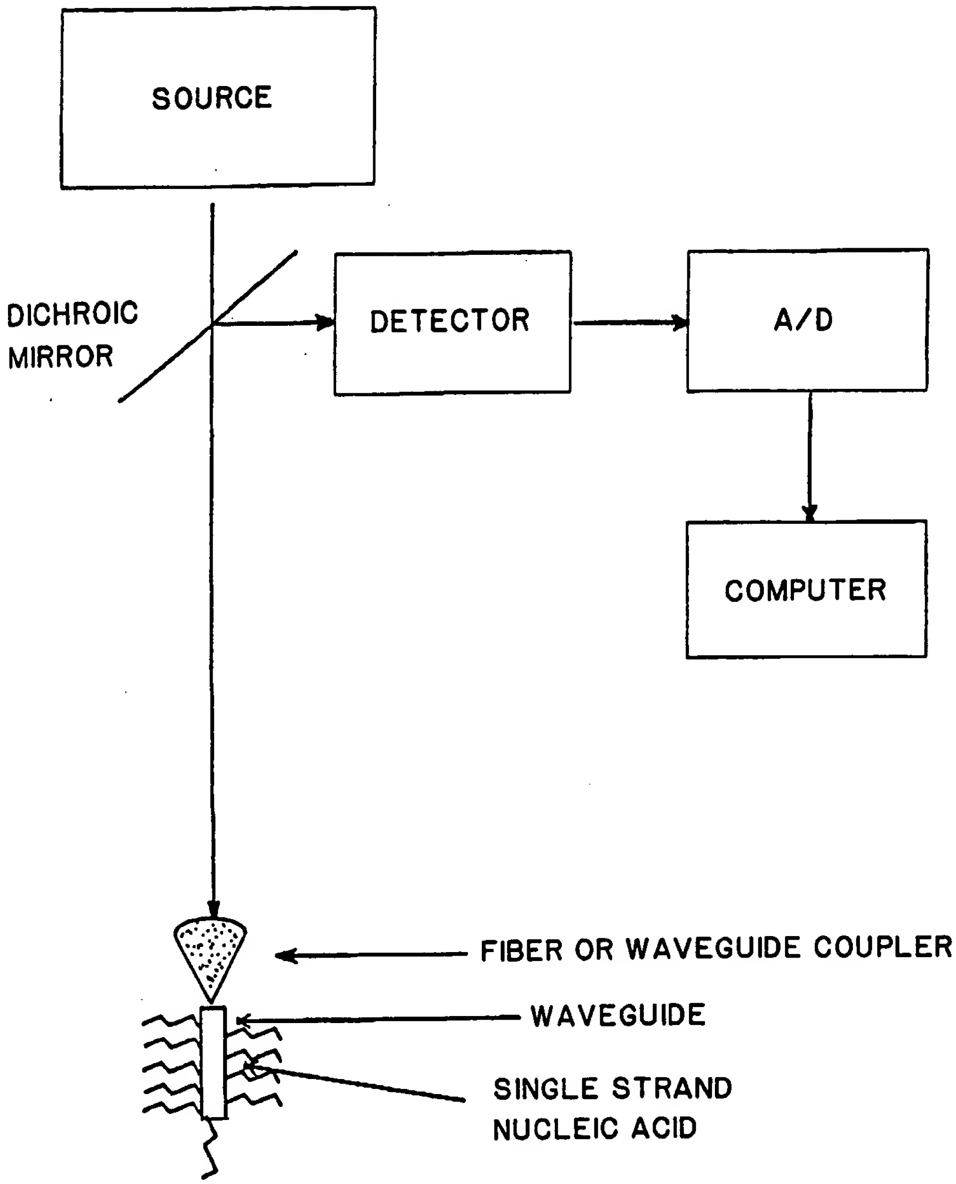

SOURCE
DICHROIC MIRROR
DETECTOR
A/D
COMPUTER
FIBER OR WAVEGUIDE COUPLER
WAVEGUIDE
SINGLE STRAND NUCLEIC ACID

FIG. 1

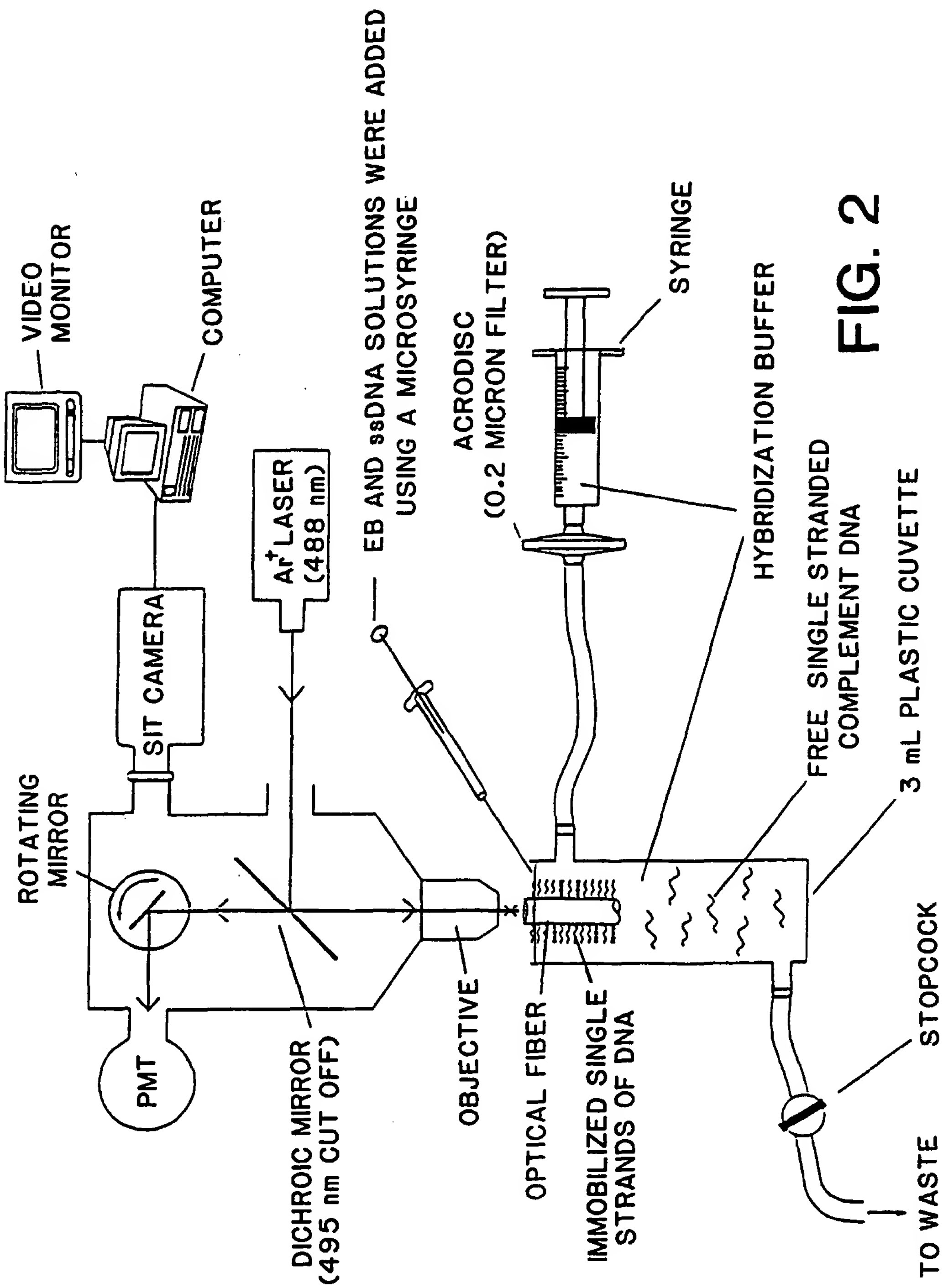

VIDEO MONITOR
COMPUTER
SIT CAMERA
ROTATING MIRROR
PMT
Ar^+ LASER (488 nm)
DICHROIC MIRROR (495 nm CUT OFF)
OBJECTIVE
EB AND ssDNA SOLUTIONS WERE ADDED USING A MICROSYRINGE
ACRODISC (0.2 MICRON FILTER)
SYRINGE
HYBRIDIZATION BUFFER
OPTICAL FIBER
IMMOBILIZED SINGLE STRANDS OF DNA
FREE SINGLE STRANDED COMPLEMENT DNA
3 mL PLASTIC CUVETTE
STOPCOCK
TO WASTE

FIG. 2

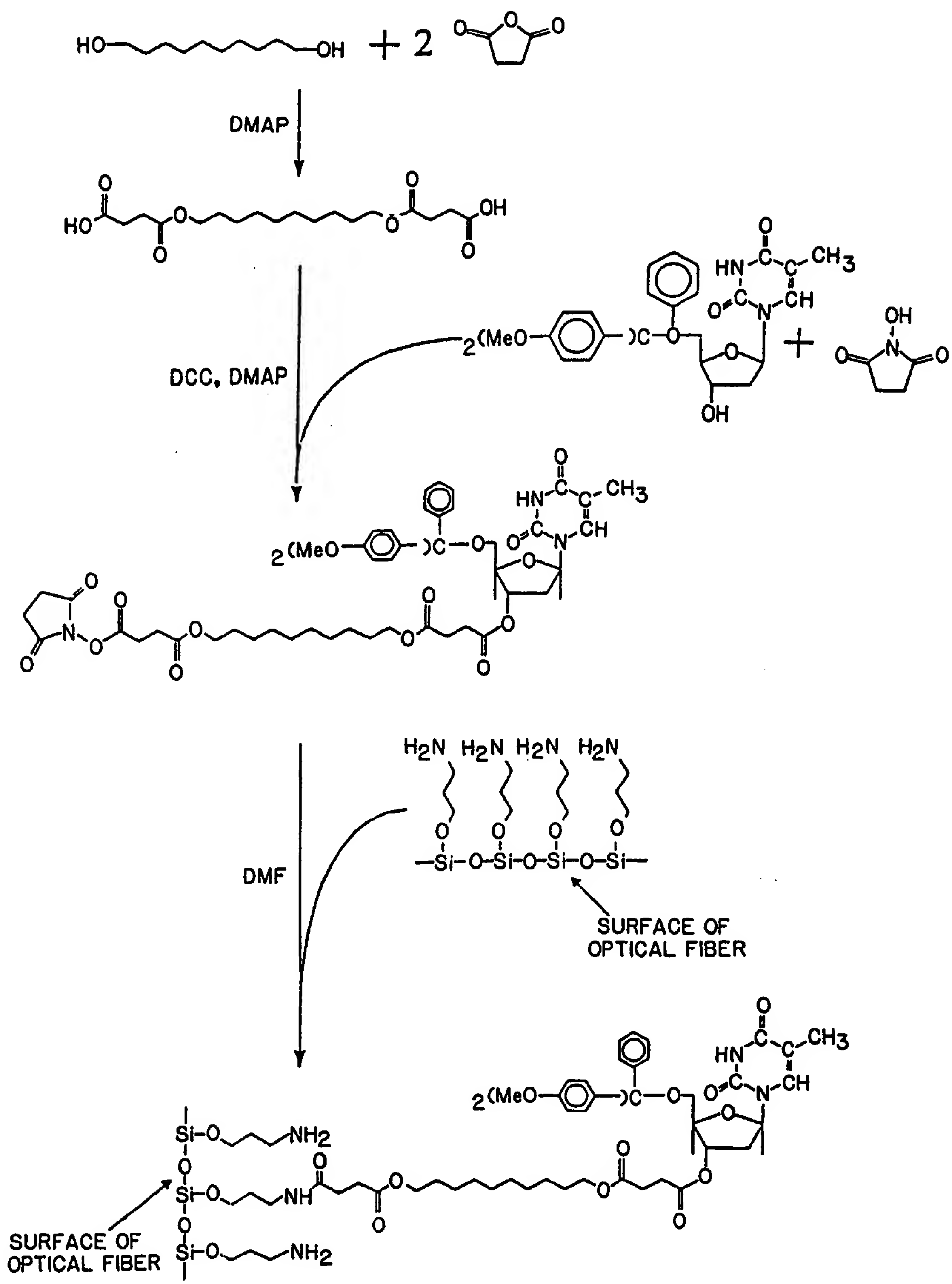
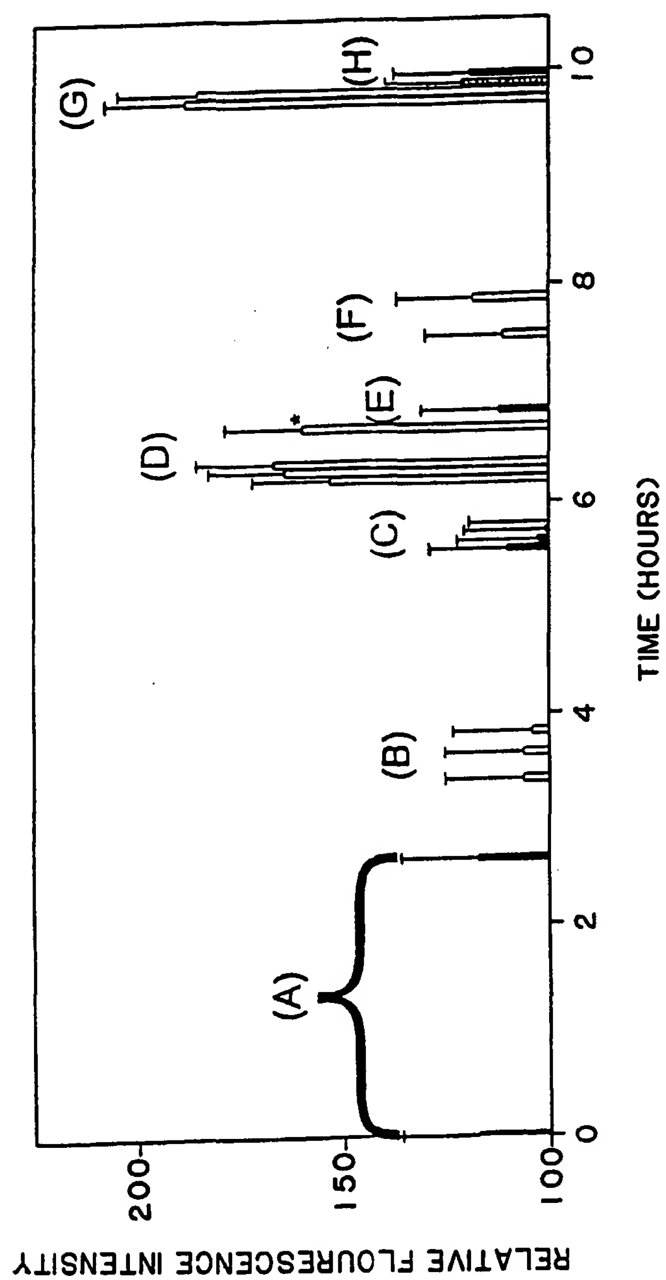

DMAP
DCC, DMAP
DMF
SURFACE OF OPTICAL FIBER
SURFACE OF OPTICAL FIBER

FIG. 3



*–THOROUGH WASHING WITH BUFFER SOLUTION

■ –ssDNA ON FIBER, NO ETHIDIUM BROMIDE ADDED

□ –ssDNA ON FIBER, TREATED WITH ETHIDIUM BROMIDE AND WASHED

▩ –FIBER TREATED WITH COMPLEMENT DNA AND WASHED, NO ETHIDIUM BROMIDE

C –dsDNA ON FIBER TREATED WITH ETHIDIUM BROMIDE AND WASHED

FIG. 4

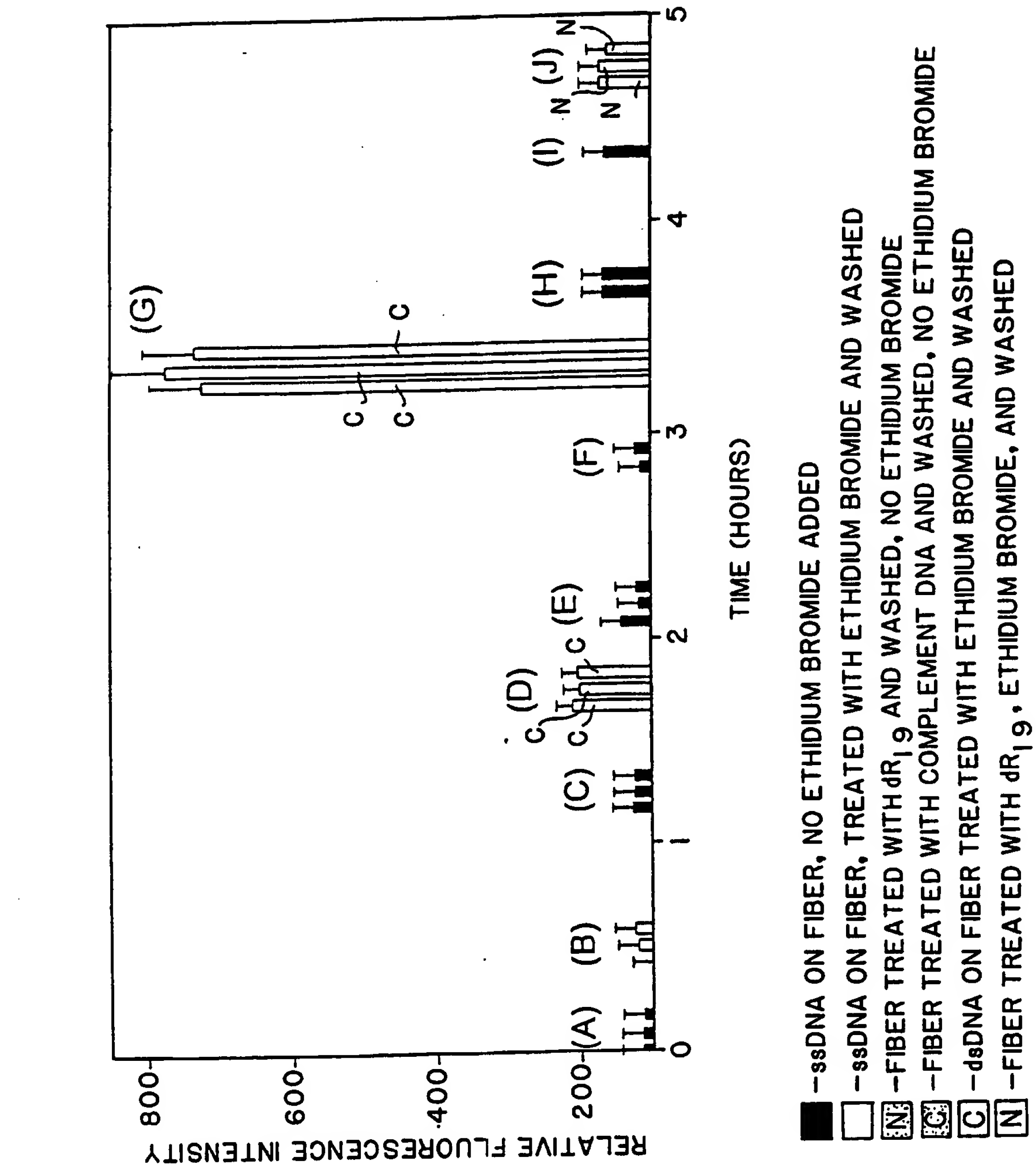

RELATIVE FLUORESCENCE INTENSITY
800
600
400
200
0
1
2
3
4
5
TIME (HOURS)
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
C
N
—ssDNA ON FIBER, NO ETHIDIUM BROMIDE ADDED
—ssDNA ON FIBER, TREATED WITH ETHIDIUM BROMIDE AND WASHED
N —FIBER TREATED WITH dR19 AND WASHED, NO ETHIDIUM BROMIDE
C —FIBER TREATED WITH COMPLEMENT DNA AND WASHED, NO ETHIDIUM BROMIDE
C —dsDNA ON FIBER TREATED WITH ETHIDIUM BROMIDE AND WASHED
N —FIBER TREATED WITH dR19, ETHIDIUM BROMIDE, AND WASHED

FIG. 5

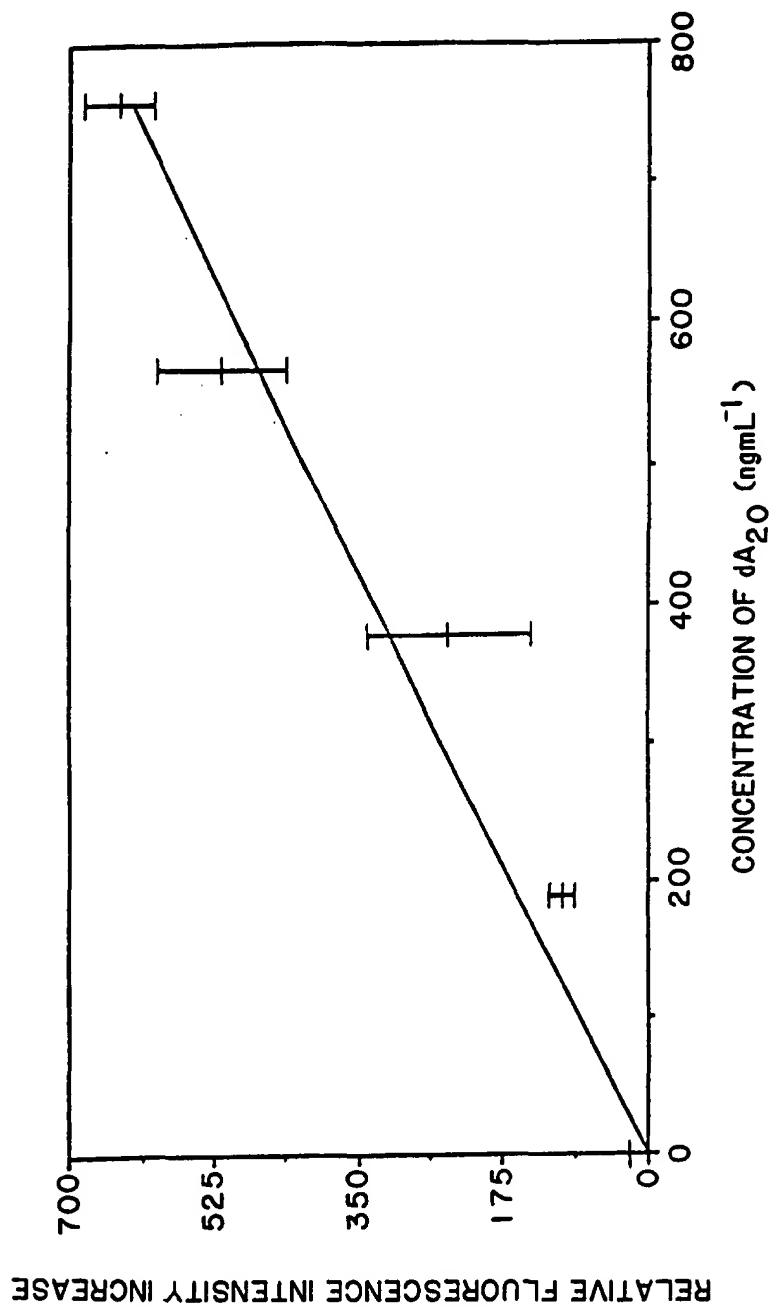

RELATIVE FLUORESCENCE INTENSITY INCREASE
0
175
350
525
700
CONCENTRATION OF dA_{20} ($ngmL^{-1}$)
0
200
400
600
800

FIG. 6

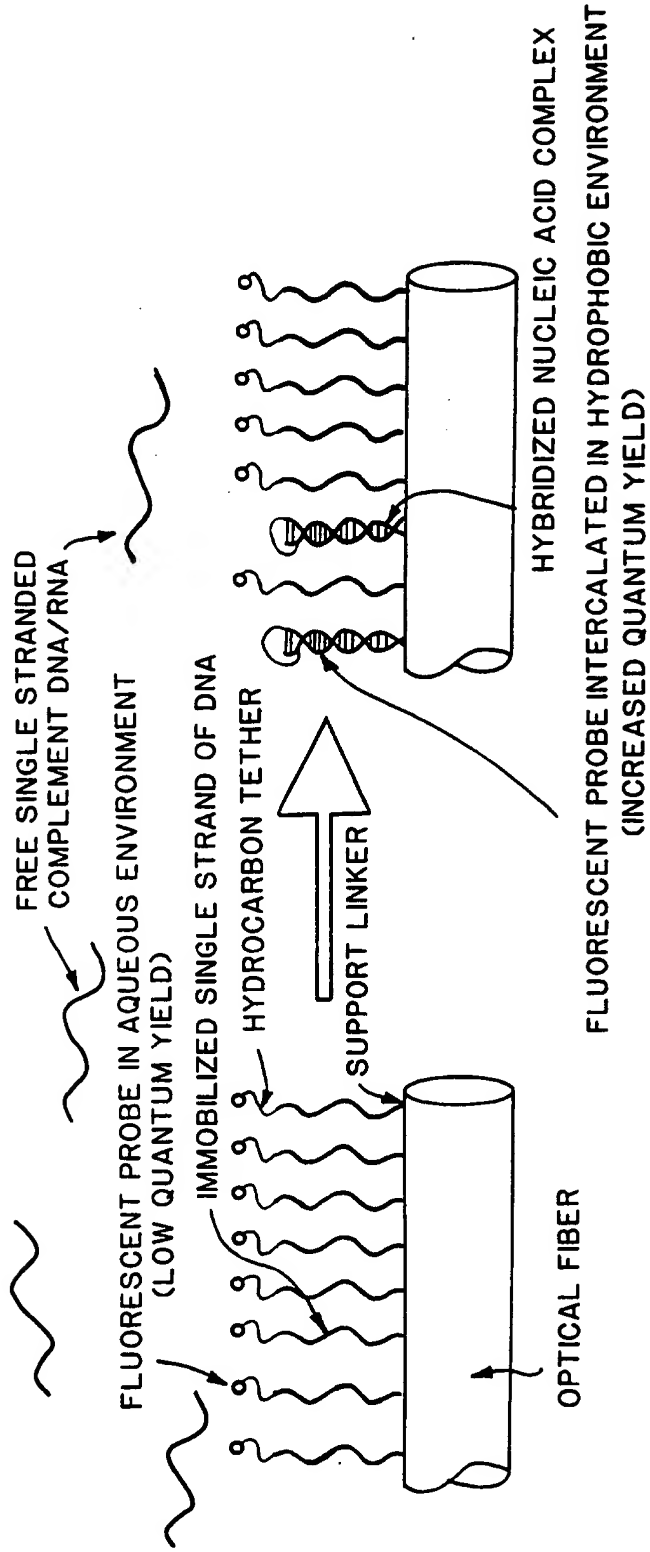

FREE SINGLE STRANDED
COMPLEMENT DNA/RNA
FLUORESCENT PROBE IN AQUEOUS ENVIRONMENT
(LOW QUANTUM YIELD)
IMMOBILIZED SINGLE STRAND OF DNA
HYDROCARBON TETHER
SUPPORT LINKER
OPTICAL FIBER
HYBRIDIZED NUCLEIC ACID COMPLEX
FLUORESCENT PROBE INTERCALATED IN HYDROPHOBIC ENVIRONMENT
(INCREASED QUANTUM YIELD)

FIG. 7A

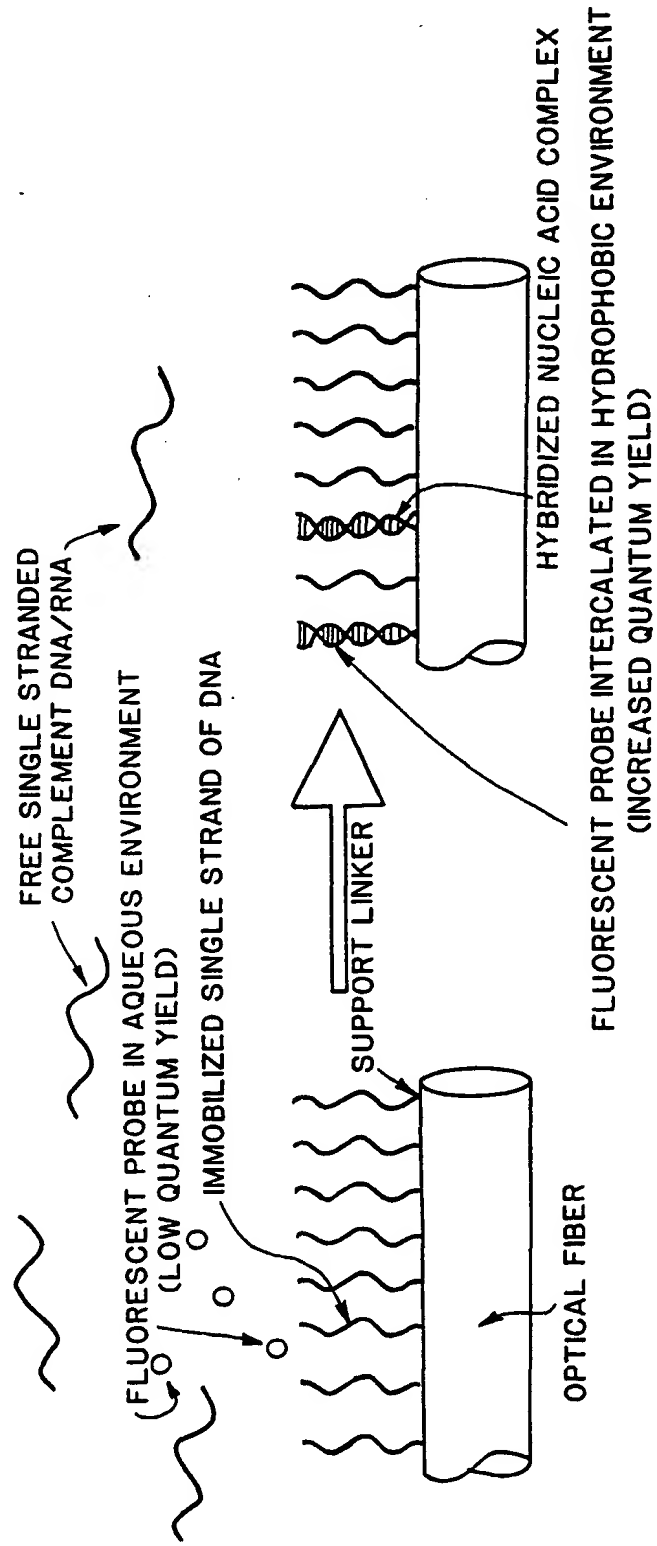
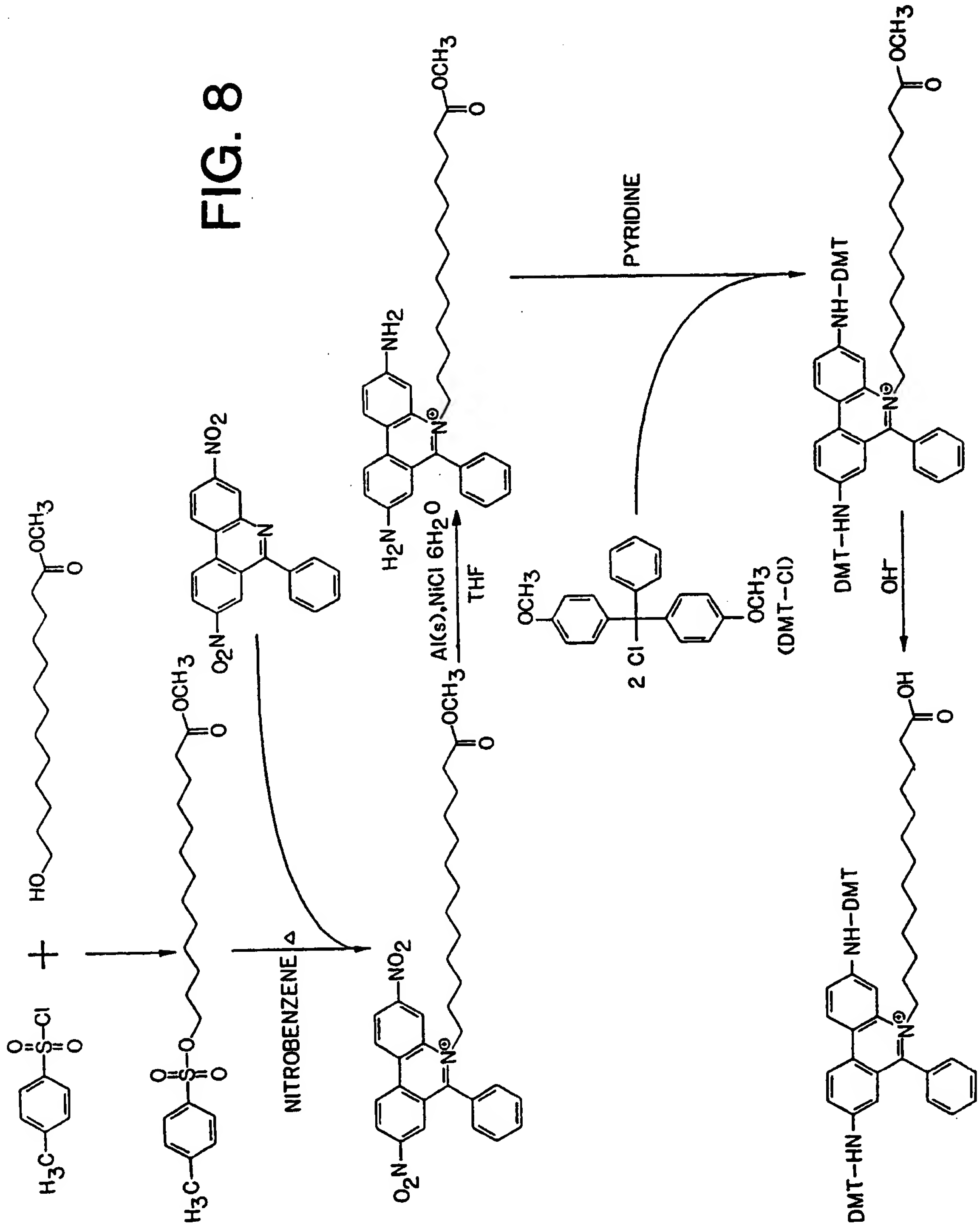
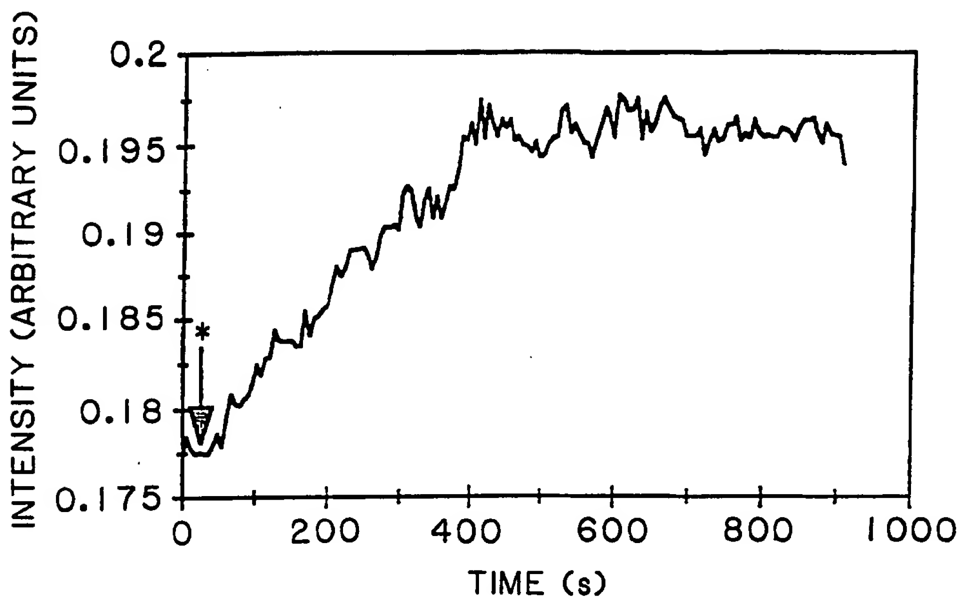

FREE SINGLE STRANDED COMPLEMENT DNA/RNA
FLUORESCENT PROBE IN AQUEOUS ENVIRONMENT (LOW QUANTUM YIELD)
IMMOBILIZED SINGLE STRAND OF DNA
SUPPORT LINKER
OPTICAL FIBER
HYBRIDIZED NUCLEIC ACID COMPLEX
FLUORESCENT PROBE INTERCALATED IN HYDROPHOBIC ENVIRONMENT (INCREASED QUANTUM YIELD)

FIG. 7B

FIG. 8


NITROBENZENE Δ
Al(s),NiCl 6H2O
THF
PYRIDINE
2 Cl
(DMT-Cl)
OH-
DMT-HN
NH-DMT



* - INJECTION OF cDNA

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03611

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 10266 (THE UNITED STATES OF AMERICA) 27 May 1993 see the whole document ---	1-36
X	EP,A,0 245 206 (BATTELLE MEMORIAL INSTITUTE) 11 November 1987 see the whole document ---	1-36
X	WO,A,93 06241 (UK. SECRETARY FOR DEFENCE) 1 April 1993 see the whole document ---	1-36
X	US,A,5 242 797 (BLOCK) 7 September 1993 see the whole document ---	1-36
A	WO,A,93 20240 (ABBOTT LABORATORIES US.) 14 October 1993 see claims 22-24 ---	1-36

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
29 June 1995	-5. 07. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03611

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 519 623 (CIBA CORNING DIAGNOSTICS) 23 December 1992 see the whole document -----	1-36

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/03611

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		GB-A,B	2190189	11-11-87
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